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With twenty-nine Plates and two hundred and forty-seven Figures

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Extension Growth in Primary Cell Walls with Special Reference to *Elodea canadensis*

BY

K. WILSON

(*Birkbeck College, London, W.C. 1*)

With Plate I and two Figures in the Text

ABSTRACT

A quantitative study of the distribution of perforations in the cellulose framework of longitudinal walls of cortical parenchyma shows that their number and spacing change in an orderly manner as the tissue grows. These perforations are considered to be pit-fields, not primarily concerned with cell-wall growth, since they do not occur in walls adjacent to intercellular spaces. The data are discussed in relation to the general problem of cell extension, and are interpreted as showing that cell-wall growth is intersusceptive throughout; tip-growth does not occur.

ELECTRON microscopy has added conspicuously to our knowledge of the structure of the cellulose fraction of primary cell walls: see for instance the work of Frey-Wyssling, Muhlethaler, and Wyckoff (1948), Muhlethaler (1950*a*, 1953), Whaley, Mericle, and Heimsch (1952), Mericle and Whaley (1953), Roelofsen and Houwink (1953). Since the chief interest of a knowledge of the structure of these walls relates to the problem of the way in which they grow, new or more clearly defined ideas of modes of primary cell-wall growth have naturally arisen from these electron microscope studies—Frey-Wyssling (1952, 1955), Frey-Wyssling and Stecher (1951), Stecher (1952), Muhlethaler (1950*b*, 1953), Houwink and Roelofsen (1954).

It is none the less important to recognize that postulates of growth mechanisms of cell-walls must be consistent not only with the sub-microscopic features of the structure of the wall—features which only the electron microscope may be able to display in detail—but also with larger-scale features of the cell as a whole and as part of a tissue, features of histology and anatomy which may be better appreciated otherwise. Some attention is drawn here to considerations of this kind.

The walls of meristem cells may contain only a small proportion of cellulose (Frey-Wyssling, 1953) and in the polarizing microscope they appear only faintly birefringent or even virtually isotropic. In cells capable of marked extension growth the birefringence is normally negative to the longitudinal direction in the growing plant member, indicating that the mean orientation of the cellulose crystallites in the wall tends approximately to the transverse. This interpretation is borne out by the electron microscope investigations, which have revealed in such cells the presence of a 'skeleton' of interwoven

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micro-fibrils of cellulose, more or less embedded in an amorphous non-cellulosic matrix; the orientation of these microfibrils may show considerable dispersion, but is nevertheless predominantly transverse.

This cellulosic skeleton of the wall is frequently not uniformly dense, but shows lenticular perforations, of the order of 0·5–2 μ in width and some 2–5 μ or more in length, their long axes commonly lying more or less transversely in the wall, in conformity with the general pattern of microfibrillar deposition. The electron microscope shows these areas to be virtually free of microfibrils, or only sparsely traversed by them, and in the polarizing microscope they appear correspondingly isotropic, though sometimes with detectable strands crossing them. There is evidence from the electron-micrographs of Muhlethaler (1953) and of Mericle and Whaley (loc. cit.) that these cellulose-free areas are the sites of protoplasmic connexions from cell to cell. They are, in short, in ordinary anatomical usage, pit-fields in the primary wall. In very young meristem cells these pit-like lenticular openings in the cellulose skeleton may be very numerous and close together, so that the cellulose fraction of the young wall has the form of a delicate lattice-like structure with transversely orientated meshes; see, for instance, the electron micrographs of Muhlethaler (1953) and the polarization photo-micrographs of Plate I herewith.

As the meristem cell grows, these cellulose-free areas become farther apart and apparently less numerous. In addition, before extension growth is complete, longitudinally orientated cellulose may be deposited, especially along the vertical edges of the cell. These 'corner thickenings', shown in detail in electron micrographs (Muhlethaler, 1950), may be visible in the polarizing microscope as regions in which the birefringences of the longitudinally and transversely orientated crystallites compensate one another. In coleoptile parenchyma these corner-thickenings may subsequently spread laterally over the longitudinal faces of the cell; in coleoptile epidermis such longitudinally orientated fibrils form the bulk of the outer wall at an early stage. Their presence clearly complicates the problem of wall growth.

In the extension growth of meristem cells having the type of primary wall structure outlined above (the so-called 'tube-structure') the essentially transverse orientation of the cellulose microfibrils, and the concomitant optical properties of the wall, appear not to be seriously disturbed, except perhaps in the rather special way described by Houwink and Roelofsen (loc. cit.). This is taken to imply that growth in area involves the insertion in the wall of new material with an orientation similar to that already prevailing—the old-established concept of intersusception. This concept has recently been restated more precisely by Frey-Wyssling and Stecher (loc. cit.) and Stecher (loc. cit.), who interpret the lenticular cellulose-free areas of the growing primary wall as regions where local loosening of the microfibrils has taken place, so allowing a local stretching of the wall to occur. New microfibrils are then woven into the regions so stretched, so that the wall structure is made good. Intersusceptive growth of this type they describe as 'mosaic growth'.

While it is clear that intersusceptive growth does occur, some types of cell, e.g. root-hairs, pollen-tubes, some fibre cells, may grow wholly or partly at their tips (Schoch-Bodmer, 1945*a* and *b*), and Muhlethaler (1950*b*) and Frey-Wyssling (1952) have concluded, from studies of coleoptile macerations, that cell-extension in unspecialized parenchyma also takes place to a considerable degree in a similar way. Cells were found which showed a relatively closely packed cellulosic skeleton in their middle parts, and a very much more open-textured structure towards their ends, and differences in the structure of the two regions were interpreted as implying a mode of cell extension by bi-polar tip growth. This differentiation of structure in the wall was only rarely seen: the reason for this, it was held, is that normally the thickening of the wall follows on closely behind the tip-extension, so that the essential nature of the cell-extension is hidden. This hypothesis of tip-growth was considered to be especially applicable to the growth of epidermal cells, where there is a thick outer wall, and Frey-Wyssling (1953), (1955), and Muhlethaler (1953), evidently consider such tip-growth to be the dominant mode of extension in elongating cells; intersusceptive growth, of a mosaic type, in which the lenticular cellulose-free areas in the wall play an essential part, is, it is postulated, important only in the early stages of elongation; the phase of tip-growth then supervenes.

If, however, this point of view is considered in the light of other evidence, certain theoretical objections to it seem to stand out. In the first place, if the cellulose-free areas in the wall are the sites of intercellular protoplasmic connexions, as the electron micrographs indicate, and if these protoplasmic connexions are important in the symplastic transport of solutes through the undifferentiated meristem, as Muhlethaler, and Scott and Lewis (1953), probably rightly, consider, then it seems unlikely that these regions should also be regions of wall growth where the cellulose network has been broken down and will in due course be made good. Secondly, it seems that if bi-polar tip-growth of meristem cells is widespread it must involve a considerable amount of gliding growth. Especially is this so in tissues where the cells are approximately prismatic with transversely orientated plane end walls; conditions very different from those prevailing in the differentiation of fusiform fibres, where some degree of intrusive- or interposition-growth may be less disturbing of intercellular relationships. As Frey-Wyssling (1950) has pointed out, extensive gliding growth must lead to loss of unity of the tissue; symplastic continuity would apparently be largely destroyed. It is relevant here to note that experimental observation of living cells in root meristems and coleoptiles does not bear out the hypothesis of tip-growth. Sinnott and Bloch (1939), working with the superficial layers of root meristems, concluded that gliding growth did not occur, and Castle (1955), investigating the growth of coleoptile epidermis by following the displacement of particles adhering to it, could find no evidence for tip-growth; cell extension was fairly uniformly distributed throughout the whole surface of the wall.

In relation to these two problems of cell extension, the mode of intersusception and the general validity of the hypothesis of tip-growth, it is of interest to examine a meristem such as that of *Elodea canadensis*, which presents certain special features of particular relevance. In the cortical parenchyma of *Elodea* the cells are arranged in rather regular longitudinal rows, and there are also considerable intercellular spaces. Furthermore the internodes are relatively short, and near the apex form a series, closely graded in length; the length of successive internodes is thus a measure of the degree of elongation of the tissue, since its initiation, at different stages of its development. Observations of the structure of longitudinal cell-walls, considered in relation to the lengths of the internodes in which they lie, might thus be expected to throw some light on the general problem of cell-wall growth, and some observations of this kind have been made.

In these observations the distribution of cellulose in the walls of meristem cells was inferred from its birefringence as seen in a polarizing microscope. The very young walls, which are only faintly birefringent (retardation of the order of 3 mμ), were rendered more easily visible by the use of an elliptic compensator to enhance the retardation of the material; critical adjustment of the compensator allowed the apparent brightness of the crystalline portion of the wall to be increased relative to that of the background. In using the polarizing microscope in this way no importance could of course be attached to the apparent extinction directions in the wall; the instrument served simply as a detector of the presence of orientated crystalline material.

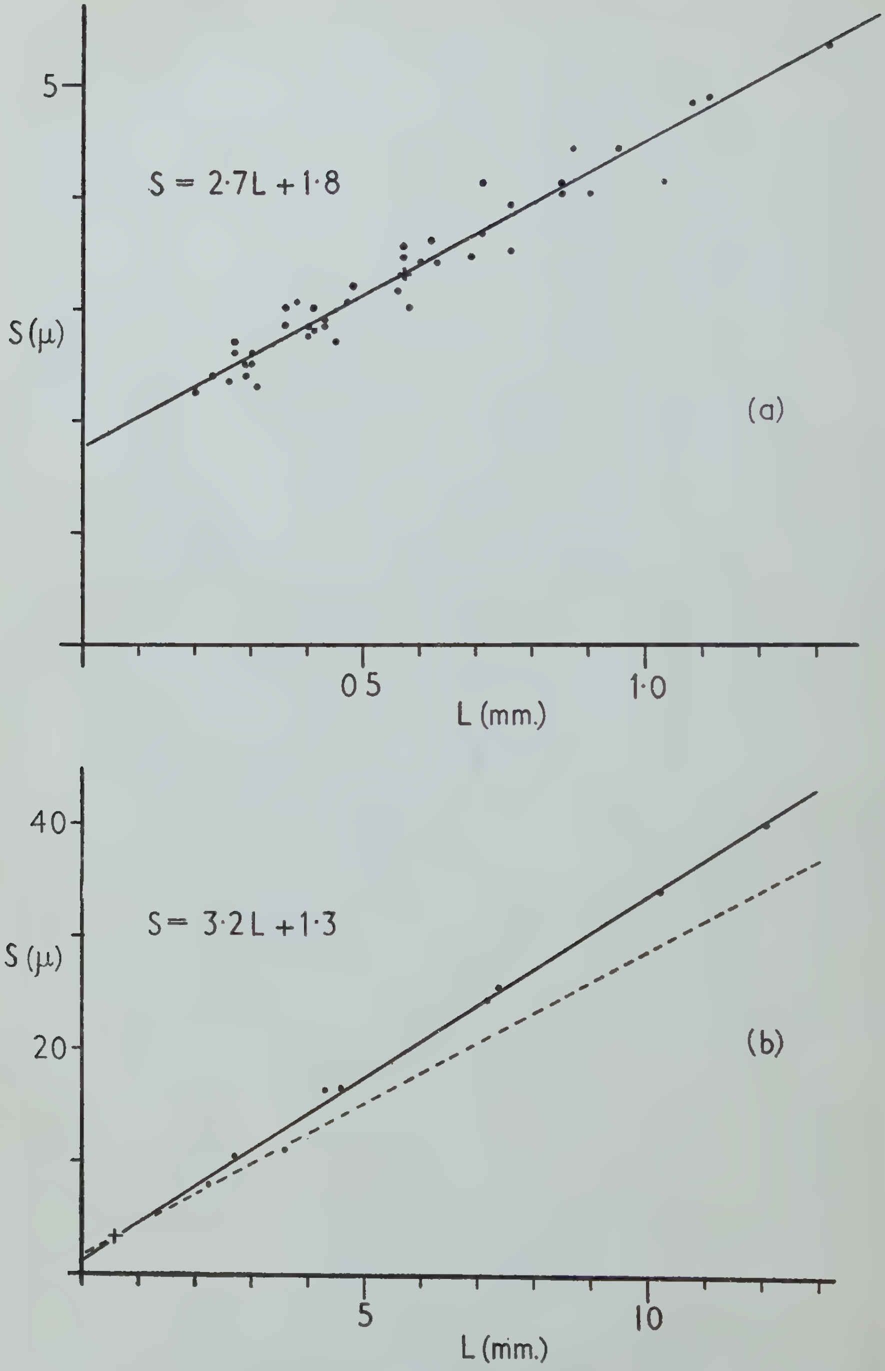
An examination of longitudinal sections of stem apices of *Elodea* in this way reveals three outstanding points. Firstly, it is noticeable that not all cell-wall surfaces show the lattice-like structure described above; many are devoid of the perforations in the cellulose framework of the wall and at the magnifications used (*c.* 600–1000) show no comparable discontinuity of structure. The birefringence of these walls is however, like the others, negative with respect to the longitudinal direction. A study of serial sections makes it clear that these uniform regions are the parts of cell-walls which abut on to intercellular spaces, and which of course in most meristems form an inconspicuous proportion of the whole. It may be added that other aquatics, such as *Glyceria aquatica* and *Hippuris vulgaris*, which have been examined, show a similar difference between the two types of wall.

Secondly, it is evident that, from its earliest appearance in the wall (judged from the faintest birefringence detectable in walls observed in face view), that the crystalline cellulose has, where it abuts on to other cells, a lattice-like structure. This may be seen in *Elodea* even in very young internodes, of length about 40 μ only, in which extension growth has barely begun. This lattice-like structure of the wall, which becomes more easily visible as the tissue grows, is in dehydrated sections, detectable only in walls seen in face view; it is rarely seen in walls observed in optical section, apparently on account of the considerable shrinkage in thickness of the wall produced by dehydration. Hand-sections of fresh, more or less mature, internodes do however show the pit-fields clearly in optical sections. Attempts to demonstrate the presence of plasmadesmata in them by the method of Crafts (1931) were unsuccessful, but it was found that sections of fresh material fixed in a saturated solution of iodine in 0·75 per cent. potassium iodide, and then swollen in 60 per cent. sulphuric acid, showed plasmadesmata as fine, brown, granular strands traversing the cell-walls in the regions of the pit-fields. The continuity of these strands with the protoplasts on both sides of the wall was sometimes seen. Similar observations on other tissues have been described by Scott and Lewis (loc. cit.).

Thirdly, a superficial comparison of cells of internodes of increasing length shows that as the longitudinal walls grow, the lenticular pit-fields become farther apart and apparently less numerous as Muhlethaler (1950*b*) and Scott and Lewis (loc. cit.) have found in other cells. This might, at first sight, seem to be because they become grown over and thus invisible, as these authors consider, though a quantitative estimate of the relationship of their spacing to the growth of the wall suggests another interpretation.

In attempting to evaluate this relationship between tissue elongation and pit-field spacing, the procedure adopted was to view longitudinal cell-walls in surface view with a squared graticule in the eye-piece of the microscope. A face of a cell-wall could thus be located centrally under a line of the graticule and the number of pit-fields in the wall which were seen to be intersected by a given length of the line could be counted. In short, the distribution of the pit-fields in the cell-wall was sampled along a line transect down the middle of a longitudinal face of the wall. That this procedure is justified is shown by the fact that the apparent mean length of the pit-fields, as seen in a polarizing microscope, does not change appreciably during growth; neither does the mean width of the cell-wall surfaces in which the pit-fields occur. Changes in their distribution along the transect may thus be related with confidence to longitudinal extension of the walls. Using sections of stem apices, the pit-field spacing along such transects was measured for internodes of lengths about 0·2–1·3 mm. In internodes shorter than about 0·2 mm. the structural features of the wall were found to be too fine and too faintly displayed for counting to be carried out, though striation of the walls was visible. The mean pit-field spacing (S), based on measurements on not less than 50 cells in each case, are shown in Text-fig. 1 *a* plotted against the corresponding internode lengths (L). Internode lengths (discounting the thickness of the nodal diaphragms), rather than cell lengths, were used as criteria of growth, since transverse cell divisions (which occur within the range of internode lengths studied) do not fundamentally affect the immediately relevant quantitative aspects of the growth of the longitudinal walls.

In addition, similar measurements were made on cells of longer internodes, of about 2–12 mm. Here the procedure adopted was to select groups of internodes of nearly similar lengths and to macerate them in 5 per cent. chromic acid. Measurements were then made on 150–300 cells in each maceration and the mean pit-field spacings so determined are shown in Text-fig. 1 *b* plotted against the corresponding mean internode lengths in each group. In



Text-fig. 1. *Elodea canadensis.* The relationship, in cortical parenchyma, between internode length (L) and mean pit-field spacing (S), measured along vertical cell-wall transects; (*a*) in short internodes, up to *c.* 1·3 mm., (*b*) in longer internodes, up to *c.* 12 mm.

The mean of all the points in (*a*), indicated by a cross, is also shown in (*b*). The unbroken lines drawn through the points are the lines of best fit in each case. The line of (*a*), extrapolated, is also shown in (*b*) as a broken line.

essence, therefore, Text-fig. 1 *a* represents the lowermost part of Text-fig. 1 *b* drawn on a larger scale, and some connexion between the two figures is furnished by the insertion in Text-fig 1 *b* of the mean of all the points in Text-fig. 1 *a*. Specimen cell-walls from internodes of various lengths are shown in the polarization photo-micrographs of Plate I.

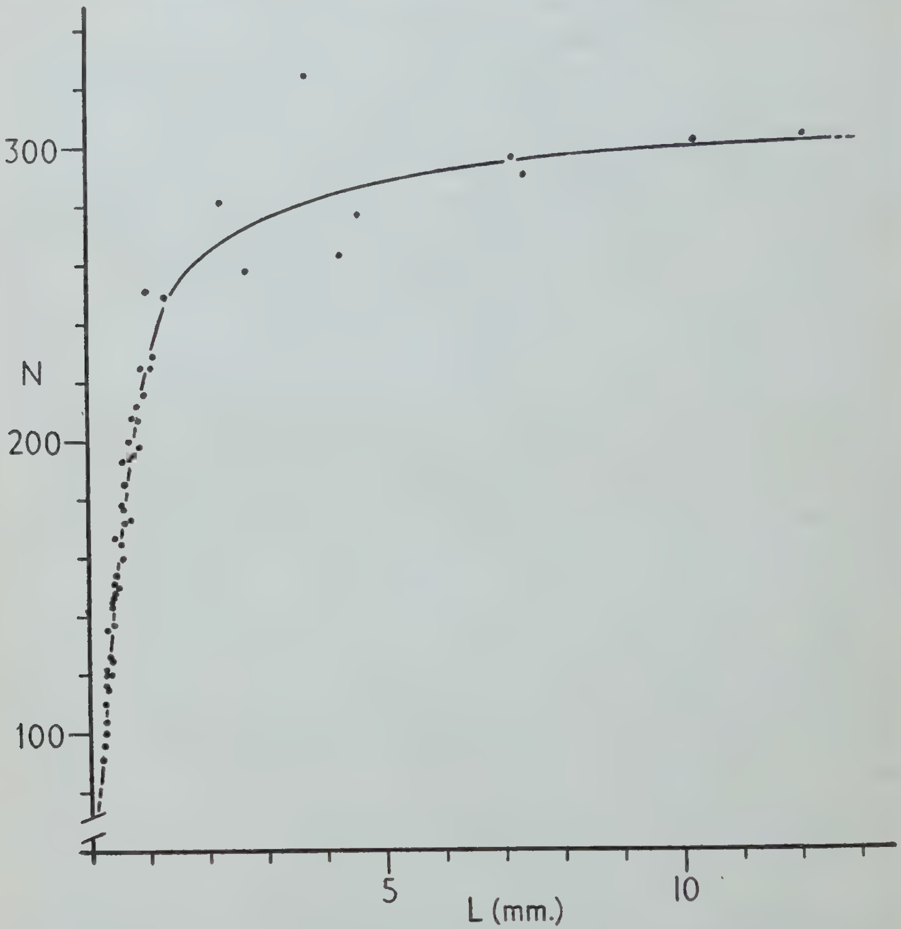
The unbroken straight lines shown in the diagrams of Text-fig. 1 are the calculated lines of best fit to the points shown in each case, and it is clear that these lines do fairly represent the trend of the relationship between pit-field spacing and internode length. This relationship is, however, apparently not linear throughout the range covered by both diagrams, since the two lines are not mathematically equivalent; the line of Text-fig. 1 *a*, if extrapolated, appears as the broken line in Text-fig. 1 *b*, the transition from one line to the other seems to occur in the range of internode lengths of about 0·8–1·0 mm.

These lines do not pass through the origin; that of Text-fig. 1 *a*, for example, if extrapolated to meet the S-axis, cuts this at about 1·8 μ, in fair agreement with the fact, already referred to, that closely spaced and faintly defined pit-fields are visible in very young internodes. This means that for short internodes, say up to about 3 mm. in length, the mean pit-field spacing is not directly proportional to internode length, though for longer internodes the approximation to direct proportionality becomes progressively closer.

This may be shown in a more illuminating way if the same data are presented in a different form. One may calculate from the individual points in the graphs of Text-fig. 1 (or from the lines of best fit to them) how many pit-fields one might expect to find intersected by a cell-wall transect extending throughout an internode of a given length. The results of such calculations are shown in Text-fig. 2, from which it is evident that the number of pit-fields along an internodal transect increases rapidly in the early stages of internodal elongation, from about 80 in the shortest internodes (*c.* 0·2 mm.) in which it was possible to make counts, and tends to a constant of about 300 in the later stages of growth (internode lengths *c.* 5–12 mm.).

From the facts (i) that the openings in the cellulose skeleton of the wall are present in cells in very short internodes, (ii) that they are always absent from walls abutting on to intercellular spaces, and (iii) that in well-extended internodes, at least, they are traversed by plasmadesmata, it seems clear that they are properly regarded primarily as pit-fields, doubtless important in plasmatic transport in the highly metabolic meristem tissue. If they play any part directly related to the growth of the wall, then a wall of a cell which abuts on to an intercellular space must grow by a different mechanism from that of a wall in contact with another cell. If this were so: if for instance a wall abutting on to an intercellular space grew by tip-growth, while one adjacent to another cell grew intersusceptively, then some considerable distortion or discontinuity in pattern of cellulose deposition about the cell would inevitably result along the line of junction between the two walls. The polarization optics of the wall offer no evidence for such discontinuity.

The alternative interpretation, that the pit-fields are not essential to cell-wall growth, is clearly the more acceptable, especially as in the later stages of wall extension the number of pit-fields along an internodal transect remains essentially constant. Here the walls are growing by intersusception *between the pit-fields*, in a manner doubtless fundamentally similar to that characteristic of the walls adjacent to intercellular spaces; it seems unnecessary to postulate the obliteration of pit-fields during growth, which Scott and Lewis, taking no account of the elongation of the tissue during development, believed to occur in their material.



TEXT-FIG. 2. *Elodea canadensis.* The relationship, in cortical parenchyma, between internode length (L) and the mean number (N) of pit-fields along a cell-wall transect extending throughout an internode, calculated from the data of Text-fig. 1.

In shorter internodes the situation is more complex. Though it may seem, from the appearance of short lengths of wall viewed in isolation, that the pit-fields become less numerous as the tissue grows, yet if their numbers are viewed in relation to the elongation of the tissue, they may be seen to increase in the early stages of internodal elongation. This increase might arise in two ways; new pit-fields might be formed by local dissolution of the wall, in the manner envisaged by Frey-Wyssling and Stecher, or alternatively, the fine strands of cellulose which may frequently be observed traversing a pit-field might themselves be involved in intersusceptive growth and so increase in width as to divide the pit-field into two or more. Which of these processes actually happens it seems impossible to determine with certainty without being able to watch the process actually taking place in living material, though the second alternative seems to be generally held to be the correct one: see for example Priestley (1930), Meeuse (1941), and Scott and Lewis (loc. cit.). It is noticeable that in older cells groups of small pit-fields may be found, their members being so disposed as to appear to be derived by division of a single larger one, which lends colour to this interpretation. The pit-fields seem in fact to be real morphological features of the primary wall (as indeed Williams, Preston, and Ripley (1955) have recently emphasized) and not merely as regions of local dissolution of the wall.

The observable distribution of pit-fields in elongating tissue also has some bearing on the hypothesis of bipolar tip-growth of parenchyma cells described by Frey-Wyssling and Muhlethaler from studies of coleoptiles. If such growth is widespread, then one might expect to find, in the walls of cells of well-grown internodes, markedly irregular pit-field distribution, with regions which, in their pitting, retain the character of the walls of much shorter internodes, showing a fine, close-meshed, lattice-like structure. This is not apparent in the cortical tissues of *Elodea*, and one must conclude that no evidence for the reality of tip-growth in this material can be adduced.

These observations, and the conclusions which have been drawn from them, are complementary to those of Wardrop (1955), described in a paper which became available as this was being prepared for publication. Wardrop finds, in *Avena* coleoptile parenchyma, that the number of pit-fields per cell remains approximately constant during the growth of coleoptiles from about 10 to 50 mm. in length. Since his measurements thus refer to coleoptiles in which cell-division had ceased, this finding is equivalent to the condition described above in *Elodea* internodes of lengths greater than about 3 mm. (see Text-fig. 2). His observations do not reveal a phase comparable to that characteristic of shorter *Elodea* internodes; it may perhaps be that in coleoptiles, as in *Elodea*, this phase is of limited duration, and that it had come to an end in the coleoptiles in which his observations were made. He concludes, from his data on pit-field distribution, that the theory of tip-growth is untenable. Concerning the mechanism of intersusceptive growth, however, he is in agreement with Frey-Wyssling and Stecher so far as to consider that the pit-fields have a special significance in this process, regarding them as centres of locally high synthetic activity. The condition in aquatic meristems, in which intercellular spaces and cell-walls lacking pit-fields are conspicuous, does not support this point of view, and seems to be consistent with it only if the wall-building activity attributed to the pit-field regions of the wall is regarded as an incidental consequence of the presence of protoplasm in these regions, rather than as one of their primary functions. It is relevant here to recall that intersusceptive growth can occur elsewhere without microscopically (or for that matter, electron-microscopically) evident discontinuity in the cellulose framework of the wall. This has been shown in cotton hairs by O'Kelley (1953) and O'Kelley and Carr (1953), in *Nitella* by Green (1954) and Green and Chapman (1955), and in various other cells by Roelofsen and Houwink (loc. cit.) and Houwink and Roelofsen (loc. cit.), whose hypothesis of cell-wall extension termed 'multi-net growth' (while probably not of universal application) takes due account of this fact.

Though in this, as in other aspects of the problem, much uncertainty still remains, it is now clear that the hypothesis of tip-growth as a widespread phenomenon in growing parenchymatous tissues must be abandoned, and furthermore that our concept of intersusceptive growth must be widened so as to admit of its reality in circumstances in which it has been considered to be impossible.

SUMMARY

In cortical parenchyma of *Elodea*, perforations (which are visible by polarizing microscope) are present in the cellulose framework of the walls between cells from a very early stage in development, but are absent from cell-walls abutting on to intercellular spaces. In more or less mature tissue they are traversed by plasmadesmata; they are considered to be pit-fields rather than loci specifically associated with wall growth.

Numerical estimates of pit-field distribution, based on line transects along vertical cell-walls, show that in internodes of lengths ranging from about 0·2 to 12 mm. the relationship between mean pit-field spacing and internode length is an approximately linear one. Pit-fields increase in number in the early stages of tissue elongation, but tend to a constant maximum in the later stages of growth. The data are discussed in relation to current concepts of cell-wall growth, and it is concluded that, in *Elodea* cortex, growth is by intersusception over the whole area of the wall, a process which does not necessarily involve microscopically visible breakdown of the cellulose framework of the wall. Localized tip-growth of the cells does not occur.

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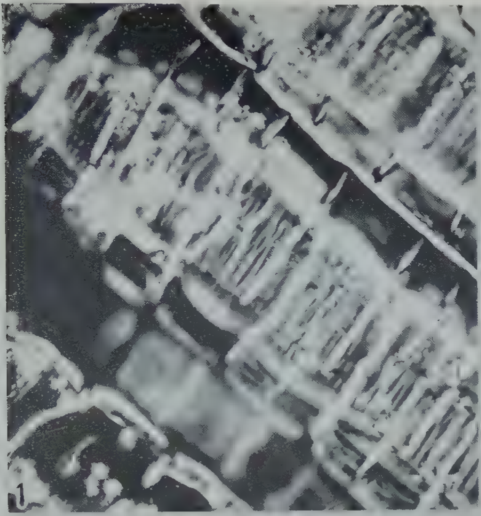
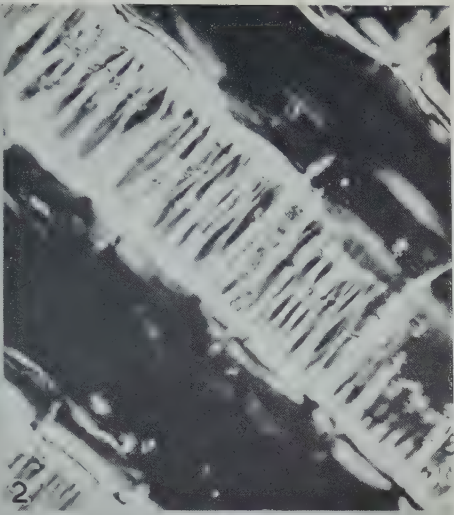
EXPLANATION OF PLATE I

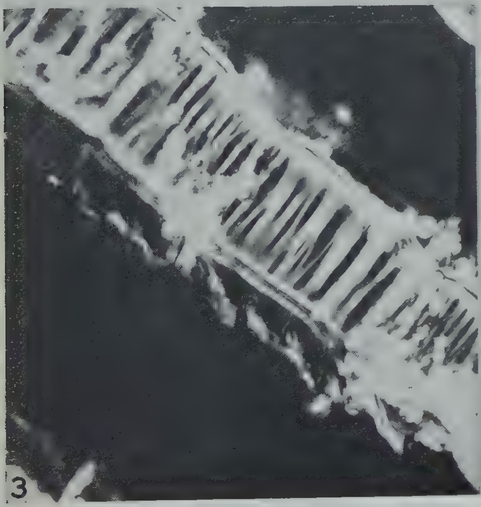
Plate I, *Elodea canadensis*. Longitudinal walls between cortical cells, from internodes of various lengths, showing the lattice-like cellulose framework; the perforations represent pit-fields. Polarization photo-micrograph, × 580. Internode lengths are: Fig. 1, 0·36 mm., Fig. 2, 0·58 mm., Fig. 3, 1·03 mm., Fig. 4, *c.* 10 mm.

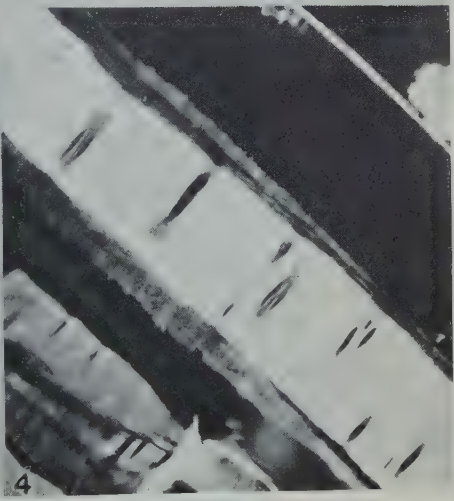
The longitudinal direction lies obliquely from upper left to lower right; dark areas between the files of cells are intercellular spaces; bright disks with dark crosses or 'figures of eight' in them are starch grains.

In Fig. 1 parts of walls abutting on to an intercellular space are also shown, rather out of focus, in the lower part of the Figure; such walls do not show the lattice-like structure of those adjacent to other cells. In Fig. 4 some pit-fields appear in groups, each group derived by division of a larger single one.









K. WILSON

Further Observations on the Effect of Carbon Dioxide on the Growth of Etiolated *Avena* Seedlings

BY

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With seven Figures in the Text

ABSTRACT

Oat seedlings when grown in 5 per cent. CO_2 show an increased growth of the mesocotyl and a reduced growth of the coleoptile. To elucidate this effect the following data, for both treated and control plants, have been obtained: (1) fresh and dry weight increases in the whole plants and in the separate organs, (2) water contents, and (3) progressive depletion of the endosperm.

The enhanced growth of the mesocotyl in CO_2 was closely correlated with dry-matter content but not with water content. The depletion of the endosperm was reduced by treatment with CO_2, and of the smaller amount of carbohydrate entering the embryo a higher proportion remained in the mesocotyl; coleoptile growth was consequently diminished. The distribution of dry matter between the coleoptile and mesocotyl must be an important factor in their reciprocal growth behaviour.

INTRODUCTION

SINCE Went's discovery of auxin and his formulation of the thesis 'Ohne Wuchsstoff kein Wachstum' (1928) it has been regarded as axiomatic that the extension growth of plants involves the participation of growth promoting substances. Their precise function has not, however, been finally elucidated; effects on cell walls and on the osmotic conditions of the protoplast, either separately or simultaneously, have been suggested (for references see review by Audus, 1949).

This paper is concerned with the rapid extension growth shown by the mesocotyl of etiolated oat seedlings when grown in an atmosphere of 5 per cent. CO_2 (Mer and Richards, 1950). As a preliminary to auxin assays primary data on the distribution of dry matter have been obtained and are presented here. Evidence for the participation of an osmotic factor associated with this growth has also been sought. It was thought that such a factor might operate if treatment with carbon dioxide induced a more rapid outflow of carbohydrate from the endosperm into the seedling. The high sugar concentration so generated in the cells would tend to be maintained, perhaps even enhanced, by the depressed respiratory activity known to obtain under these high CO_2 conditions (Mangin, 1896, Willaman and Beaumont, 1928). If these circumstances were realized, then the preferential growth of the mesocotyl might be explained in terms of its proximity to the carbohydrate supply.

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The primary aim of this investigation therefore was to obtain the data necessary to reach a decision on this point.

MATERIALS AND METHODS

Svalof 'Victory' oats were used for the experiment. The plants were grown as previously described (Mer, 1953) in light-tight tin boxes in a thermostat regulated at 24° C. Each tin contained six sets of 57 plants; the controls in one tin were continuously supplied with air which after having been filtered through activated carbon was saturated with water vapour, while in the second tin the plants were treated similarly except that carbon dioxide was injected into the airstream from a cylinder to give a final concentration of 5 per cent.

The seeds were dehusked and weighed individually; those weighing between 30 and 40 mg. were selected. Their weights were recorded and they were planted in numbered seed-holders, so that subsequently each plant when measured could be referred back to the seed from which it had grown.

Two sets of plants, one from the corresponding position in each box, were removed in darkness for measurement each day from the 3rd to the 8th day after planting. The lengths of the mesocotyl, coleoptile, and leaf were recorded and the plants were randomly assorted into groups of 10, which thereafter were treated as units to provide the following data: (*a*) dry weight of 10 endosperms (including scutellum), (*b*) total fresh weight of the 10 plants, (*c*) fresh weights of mesocotyls, coleoptiles, leaves and roots (i.e. 10 of each derived from the 10 plants) and (*d*) the dry weights of these separate samples. The material was dried for 24 hours at 75° C. in an air oven and stored in a vacuum desiccator over calcium chloride until weighed. From these data the water content (per mg. dry weight) of the whole samples and of the separate parts of the seedling were calculated. The details appertaining to the determination of the outflow from the endosperm will be dealt with at the appropriate place in the text.

Two experiments giving essentially similar results were carried out, but the data presented refer to one only in which five replicate samples (each of 10 plants) were obtained daily for both treated and control plants.

EXPERIMENTAL RESULTS

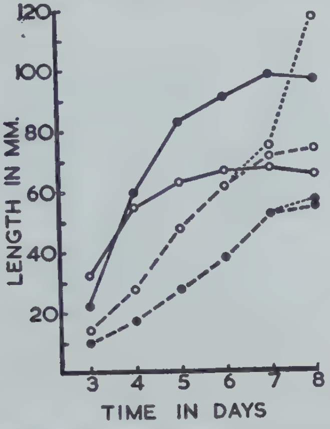
1. *The growth of the plants*

The growth of the plants is shown in Text-fig. 1 in which the mean length of 50 plants is plotted against time. It will be seen that the treatment reduced the growth of both mesocotyls and coleoptiles up to the 3rd day but that subsequently while the mesocotyls extended very rapidly and finally were very much longer than the controls, the coleoptiles were persistently depressed.

The leaves of the control plants emerged slightly earlier and after emergence they grew faster than did those of the treated plants. The emergence of the leaves implies the termination of coleoptile growth and consequently treatment with CO_2 caused a permanent reduction in its length.

2. *Fresh weight*

i. Two estimates of the total fresh weight in gm. of the 50 plants are shown in Table I; the first was obtained prior to dissection of the plants while the second was obtained by adding the weights of the separate parts. The percentage differences are also recorded, and these represent the losses which occurred during handling. They vary between 2·68 per cent. and 5·16 per cent., but show no consistent bias.



TEXT-FIG. 1. The effect of additional carbon dioxide on the growth of mesocotyls, coleoptiles, and leaves.

○——○——○ mesocotyls }
○ – – ○ – – ○ coleoptiles } controls—open circles.
○----○----○ leaves } 5 per cent. CO_2—full circles.

TABLE I

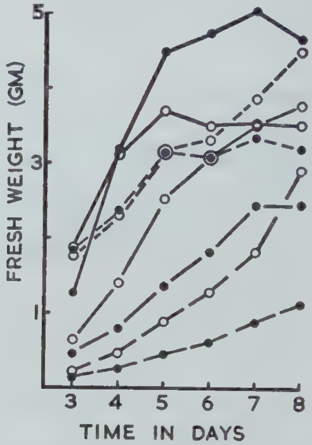
Daily Fresh Weight (gm.) of 50 Plants (without Endosperm)

Days		3	4	5	6	7	8
Controls . .	(*1*)	4·66	7·49	10·72	11·81	13·19	15·36
	(*2*)	4·50	7·29	10·24	11·22	12·67	14·60
Per cent. loss .		3·46	2·68	4·43	4·97	3·92	4·96
Treated . .	(*1*)	3·90	6·96	9·70	10·65	12·12	11·84
	(*2*)	3·70	6·62	9·42	10·20	11·65	11·35
Per cent. loss .		5·16	4·77	2·96	4·30	3·91	4·10

(*1*) Weight of plants before dissection.
(*2*) Sum of the separate weights of the mesocotyls, coleoptiles, leaves, and roots.

The data show that the fresh weight of the treated plants was always less than that of the controls.

ii. The fresh weights of the individual parts of the seedling are shown in Text-fig. 2, in which the weight of 50 segments is plotted against time. The data follow the pattern shown for lengths in Text-fig. 1. Thus the treated mesocotyls were lighter than the controls on the 3rd day but subsequently increased very much in weight and were finally much heavier. The coleoptiles and leaves were depressed throughout; root weight was depressed below controls on the 6th, 7th, and 8th days.



TEXT-FIG. 2. Effect of additional carbon dioxide on the fresh weight of the different parts of the seedling (50 segments).

○———○———○ mesocotyls
○ —— ○ —— ○ coleoptiles
○— —○— —○ leaves
○ - - - ○ - - - ○ roots
controls—open circles.
5 per cent. CO_2—full circles.

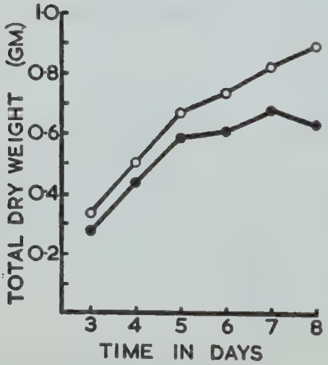


FIG. 3

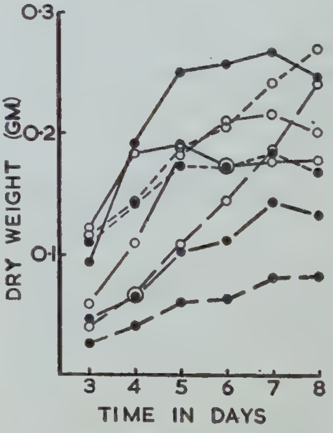


FIG. 4

TEXT-FIG. 3. Effect of additional carbon dioxide on the total dry weight of 50 plants.

TEXT-FIG. 4. Effect of additional carbon dioxide on the dry weight of the different parts of the seedling (50 segments).

○———○———○ mesocotyls
○ — ○ — ○ coleoptiles
○— —○— —○ leaves
○ - - - ○ - - - ○ roots
controls—open circles.
5 per cent. CO_2—full circles.

3. *Dry weight*

Text-figs. 3 and 4 show respectively the total dry-weight data and that of the separate parts. The total dry weight of the treated plants was consistently less than that of the controls, while the weights of the segments conform with the fresh-weight data.

4. *Water content*

i. Whole plants. The water content of the daily samples calculated from the total and summed fresh weights ((1) and (2) of Table I) are shown in Table II. These results show that the treated plants had a higher water content than the controls, and that apart from the 3rd-day sample (in Table II (*b*)) the variation in loss during handling did not eliminate this treatment effect.

TABLE II

Water Content (in mg. H_2O/mg. dry weight) of whole samples

(*a*) *Weighed Total Fresh Weight*

Day	3	4	5	6	7	8
Controls . .	12·90	13·88	15·00	15·07	15·05	16·26
Treated . .	13·03	14·81	15·53	16·57	16·92	17·77

(*b*) *Summed Total Fresh Weight*

Day	3	4	5	6	7	8
Controls . .	12·42	13·49	14·29	14·27	14·42	15·41
Treated . .	12·30	14·05	15·04	15·81	16·22	17·00

ii. Water content of the separate parts of the seedling. The data referring to the mesocotyls, coleoptiles, leaves, and roots separately are shown in Table III together with a résumé of the analysis of variance.

TABLE III

Water Content of the Mesocotyls, Coleoptiles, Leaves, and Roots (in mg. H_2O/mg. dry weight): totals of the 5 replicate samples

Days	3	4	5	6	7	8
		Mesocotyls				
Controls . .	71·9	79·9	91·6	95·6	94·6	93·0
Treated . .	62·9	78·4	84·4	86·5	88·8	88·7
		Coleoptiles				
Controls . .	48·9	59·0	64·2	69·9	75·9	88·4
Treated . .	43·0	55·9	61·1	76·0	79·8	86·7
		Leaves				
Controls . .	24·3	30·5	35·9	39·3	43·5	55·1
Treated . .	20·7	26·2	31·8	42·1	49·1	62·5
		Roots				
Controls . .	70·7	75·0	78·5	75·1	74·5	77·7
Treated . .	78·2	78·9	85·6	83·9	85·6	89·5

Analysis of Variance

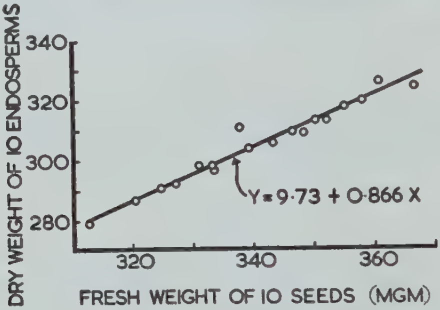
		Mean Squares			
	DF	Mesocotyls	Coleoptiles	Leaves	Roots
Treatment .	1	22·85*	0·24	0·25	42·33*
Time . .	5	37·39*	90·33*	68·48*	4·47*
Interaction .	5	0·87†	2·14*	2·79*	0·85†
Error . .	48	0·26	0·20	0·10	0·32

* Significant at 1 per cent. † Significant at 5 per cent.

The water content of the mesocotyls was consistently reduced by treatment while for the roots the situation was exactly reversed. The water content of the coleoptiles and leaves was variable; on the 6th and 7th days it exceeded that of the controls while on the other days it was reduced. The positive and negative effects cancelled to give a net absence of response. Because of the switch-over, however, there was a significant interaction. The reason for this change in response is not known; it was also shown in a previous experiment in which, for the coleoptiles, treatment reduced the water content on the 3rd, 4th, and 5th days, but raised it on the 6th, 7th, and 8th days. The positive and negative effects did not cancel, however, and there was a net reduction in water content. The leaves behaved slightly differently, there was a uniform depression in water content except on the 7th day when the values were equal.

5. *Depletion of the endosperm reserves*

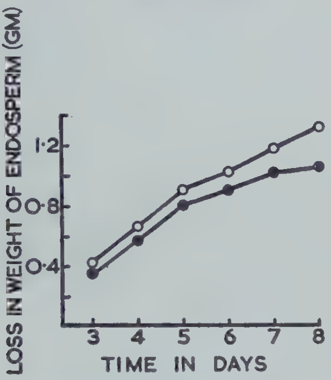
To estimate the outflow of carbohydrate from the endosperm it was necessary to know, for each replicate group of 10 endosperms, their initial and final dry weights. The final dry weights were obtained directly by weighing the endosperms which had been removed from the plants and dried. The initial dry weights could not, of course, be obtained directly but had to be estimated from the fresh weight of the seeds. The method of recording employed permitted the reference of each plant to the seed (whose weight was known) from which it had grown, and consequently as the 10 plants comprising each group were noted so the weights of their seeds were automatically specified. The total fresh weight of each of the 60 groups was thus obtained by adding the weights of the individuals and when the experiment had been completed 18 groups of 10 seeds were selected whose weights were distributed over the range of those actually used in the experiment. After weighing them the embryos were removed and the dry weights of the endosperm (with scutellum) were found. The regression of endosperm dry weight on the initial weight of seed was calculated and is shown in Text-fig. 5.



TEXT-FIG. 5. Regression of dry weight of endosperms (with scutellum) on initial weight of seeds.

It will be seen that the observations lie quite closely to the calculated line of best fit. In a previous experiment the weights of the 10 seeds varied between

200 and 300 mgm., and the slope of the regression line was 0·868, as compared with 0·866 in Text-fig. 5. Evidently these two sets of observations would lie on the same straight line. This means that over the fresh-weight range examined the dry weight of the endosperm forms a constant proportion of the fresh weight of the seed. The embryo must do likewise. It may be inferred, therefore, that the size of the embryo is adjusted to the size of the seed. The equation of the line—$y = 9{\cdot}73 + 0{\cdot}866\,x$ where y is the dry weight of the endosperm plus scutellum and x is the initial weight of the seeds—was now used to calculate the endosperm dry weight of the groups of seeds actually used in the experiment. The weight losses, i.e. outflow of reserves into the seedling, were then obtained by subtraction. The result is shown in Text-fig. 6; treatment with carbon dioxide reduced the outflow of carbohydrate from the endosperm.



Text-fig. 6. Loss in weight of endosperms in air (open circles) and with additional carbon dioxide (full circles).

Discussion

The data presented in this paper provide an unequivocal answer to the question originally posed. It is quite clear that treatment with carbon dioxide slows down the flow of carbohydrate from the endosperm into the seedling. This result was indicated by the total dry-weight data (Text-fig. 3), for had the suggestion been correct then the total dry weight of the treated plants would inevitably have exceeded that of the controls, but this was not so. Thus far the data are consistent, indicating that the method of deriving the outflow from the endosperm is not seriously in error.

Thus, in the controls a high sugar intake is associated with a higher respiratory activity while in the treated plants there is a reduced sugar intake accompanied by a reduced respiratory activity. It would not be unreasonable to assume that the free sugar level in the two sets of seedlings was approximately the same (although this requires experimental verification) and it is consequently unlikely that an osmotic factor is associated with or causes the rapid extension growth of the mesocotyl.

Van Overbeek (1944) and Hackett (1952) have both concluded that the enhanced water-uptake shown by storage tissue following treatment with auxins (both synthetic and IAA) cannot be accounted for in terms of an altered osmotic status of the constituent cells.

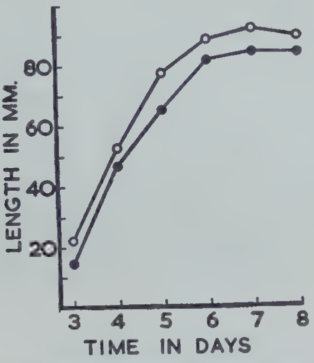
Neither can the rapid elongation of the mesocotyl in CO_2 treated seedlings be correlated with water content for, as shown in Table III, this is consistently less than that of the controls, while its length is successively less than, equal to, and finally greater than the control measurements. Furthermore from the 3rd to the 5th day, when the mesocotyl was extending rapidly, its water content was reduced below that of the controls; the coleoptile on the other hand was growing slowly and its water content was likewise depressed below that of the controls: hence there can be no causal relationship between water content and elongation.

The distribution of growth between the mesocotyl and coleoptile may now be considered and we have to deal with both a promotion and a reduction of growth. Promotion of growth following treatment with carbon dioxide has been reported by Braun (1932) and by Ruck and Bolas (1954) who found that the increased height of their plants was associated with a reduction in dry matter. In *Avena*, however, there is no doubt that the promotion and depression of growth are directly correlated. The dry-weight data (Text-fig. 4) show that under the high CO_2 conditions the mesocotyl absorbs a disproportionately large part of the reduced quantity of carbohydrate flowing into the seedling. Consequently the depressed growth of the coleoptile might reasonably be attributed to partial starvation, without the need to postulate any change in its auxin metabolism.

The problem therefore hinges upon the changes induced by CO_2 treatment in the mesocotyl and at present only indirect evidence is available on this point. The wheat seedling does not form a mesocotyl in darkness, nor does it do so when exposed to carbon dioxide. This circumstance presents the opportunity of observing the effect of CO_2 on the extension of an auxin-controlled organ, uncomplicated by the presence of a mesocotyl and in close proximity to its food-supply. Text-fig. 7 shows the result. There is a persistent depression with no indication whatsoever of a promotion of growth. This may be due to a change either in auxin or in food-supply. In the absence of auxin assays only the latter can be discussed with certainty. The average reduction in growth of the wheat coleoptile was 11–12 per cent. In the oat data the average reduction in outflow from the endosperm was 16 per cent. If the outflow from the wheat endosperm was correspondingly reduced a depression in growth, as observed, would result.

There is ample evidence that in the oat coleoptile cell division ceases after 2–3 days and that all further growth is by cell extension only; for this reason the oat coleoptile is regarded as the ideal tissue for cell extension studies, and there is no reason to believe that the wheat coleoptile behaves differently. Clearly, therefore, treatment with carbon dioxide reduces the length of an organ whose growth depends on cell extension only, and it may be inferred that the rapid growth of the mesocotyl is not primarily due to this process. In the mesocotyl an organized meristem is present in the nodal region (Boyd and Avery, 1936) and if the effect of treatment were to maintain the activity of thie meristem the growth phenomena recorded in this paper would ensue.

The immediate effect of CO_2 is to depress growth and this is shown by the 3rd-day sample. It is suggested that active cell division has been taking place and that there is present, in consequence, a larger number of cells all of which passing through a grand period of growth produce the observed extension. In so doing they utilize the available carbohydrate for the synthesis of cellulose, which leads to the increase in their dry weight and to the simultaneous deprivation of the coleoptile and plumule, whose growth is correspondingly reduced.



TEXT-FIG. 7. Growth of wheat coleoptile in air (open circles) and with additional carbon dioxide (full circles).

Data on cell numbers and lengths are required to reach a decision on this suggestion and this investigation is currently in progress; estimates of auxin content will be required to complete the analysis.

ACKNOWLEDGEMENTS

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Keto-acids in the Alga *Chlorella*

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ABSTRACT

The intracellular concentration of pyruvic and α-ketoglutaric acids in *Chlorella* when grown under various conditions has been investigated. The keto-acids were assayed after quantitative elution of their 2,4 : dinitrophenylhydrazones, chromatographically separated.

Very small amounts of keto-acids are found in *Chlorella* when grown autotrophically. The level was much higher under heterotrophic conditions and in nitrogen starvation. Further, with nitrate the level was considerably higher than if ammonia was supplied. In the presence of glucose this differential effect occurred with pyruvic acid, but the level of α-ketoglutaric acid appeared to be stabilized. Possible interpretations of these facts are discussed.

INTRODUCTION

THE important position occupied by pyruvic acid in the interlinked metabolic systems of glycolysis, aerobic oxidation, fat and protein metabolism, has been reviewed by many authors, notably Krebs (1943), Barron (1943), and Stoltz (1945). Little work has been done with plant tissues beyond the demonstration of the presence of keto-acids in a number of species of higher plants (James and James, 1940; Virtanen, Miettinen, and Kunttu, 1953; Towers, Thompson, and Steward, 1954; Fowden and Webb, 1955), and other than the path of carbon in photosynthesis, practically nothing on algae. The assumption of the citric acid cycle as a universal feature of aerobic organisms, of course, presumes the presence of the keto-acids, pyruvic, α-ketoglutaric, and oxalacetic. Work on the demonstration of this enzyme system in green plants has been done by Bonner and Wildman (1946), Bonner (1948), Danforth (1953), Davies (1953), Millerd (1953), Smillies (1955), Laties (1953), amongst others.

Little is known, however, about the variation in intracellular concentration of keto-acids in plant tissues, except for the work of Barker and Mapson (1953) on potato tubers, and nothing on the effect of the nitrogen source, although Wirth and Nord (1942), and Nord and Mull (1945) discuss the effect of nitrate on the accumulation of pyruvate in the growth medium of *Fusarium*.

Clark (1936), Bonner (1944), Vickery, Pucher, Wakeman, and Leavenworth (1940) studied the differential effect of nitrate and ammonia on intracellular plant acids. Due to lack of sensitive estimation techniques, material has often been chosen from the succulents, which have unusually high concentrations of acids. Only with the introduction of chromatographic techniques has it been

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possible to assay small quantities of the order of 25 μg. and thus extend the range of organisms studied.

The keto-acids, with their generally assumed role as intermediates in carbohydrate metabolism and as amino-acid precursors, form an important link between carbohydrate and nitrogen metabolism; this fact, taken together with the results of Syrett (1953), and Moses (1954), showing that the rate of respiration of *Chlorella* and *Zygorrhynchus* was closely dependent on the source of nitrogen, was of sufficient interest to warrant an investigation of the keto-acids of *Chlorella* and the effect of nitrogen supply on their concentration.

Material and Methods

Growth of cultures. The alga used was a strain of *Chlorella vulgaris*, used by Syrett (1951). It was grown in pure culture in the apparatus previously described (Millbank, 1953), the medium used being that described by Syrett (1951). Growth was continued for 4–5 days, the yield being approx. 2–3 g. dry wt. The cells were harvested by centrifuging at 950 × *g* for 5 min.; the centrifuged cells were then washed by repeated centrifuging from distilled water and finally resuspended in the appropriate experimental medium.

Measurement of intracellular keto-acids. Paper chromatography was used, the method adopted being developed from that of Cavellini, Frontali, and Toschi (1949).

An aliquot of algal suspension containing a known weight of cells was centrifuged and the cells deproteinized for 16 hours at 0–2° C. with fresh metaphosphoric acid, the volume and strength of which was arranged to give a final concentration of *c.* 5 per cent. The denatured material was centrifuged off, and the supernatant, which had a volume of 10–15 ml., retained. To this, 2·0 ml. of 0·1 per cent. (w./v.) 2, 4 : dinitrophenylhydrazine (DNPH) in 2N HCl was added, and the solution kept at 37° C. for 30 minutes. The resulting DNPH derivatives of the ketone substances, together with unchanged reagent, were then extracted with four 4 ml. portions of ethyl acetate. The upper layers were withdrawn with a Wright capillary pipette and bulked. Acidic derivatives were then extracted from the ester solution by four 2 ml. portions of 10 per cent. (w./v.) sodium carbonate solution. A further extraction from this alkaline solution with 2 ml. of ethyl acetate ensured maximum removal of unchanged reagent. The alkaline solution of keto-acid hydrazones was then rendered just acid with conc. HCl at 0° C. and the acid solution extracted with three 2 ml. portions of ethyl acetate. These were bulked and evaporated to dryness with a stream of cold air. The solid residue was taken up with 0·5 ml. of 0·5 N ammonia solution, and an aliquot, usually 50 or 100 μl., applied as a spot to Whatman No. 3 filter paper in the usual way. The descending chromatogram was run for upwards of 16 hours, using 50 : 50 butanol : 6 per cent. ammonia as solvent (Fincham, 1953). Sharply defined spots of keto-acid derivatives were obtained. Pyruvic acid gave rise to two spots as the majority of other authors have found. Rf. values: α-ketoglutaric acid, 0·16; oxalacetic acid, 0·20; pyruvic acid 0·45 (and 0·55). The values vary slightly with the concentration of ammonia. After running, the paper is dried in air and the areas containing keto-acid derivatives cut out and the derivatives eluted by soaking for one hour in 4 ml. of 10 per cent. (w/v) sodium carbonate. With pyruvic acid, only the slower-moving spot was eluted. Paper blanks were also processed. 3 ml. of the eluate were taken, 0·5 ml. 3N sodium hydroxide solution added and the optical density read off at 540 mμ using a 'Unicam' SP 500 spectrophotometer. The optical density was linear between 10 and 80 μg. acid. Results were consistent from week to week, to within ±8 per cent. A calibration curve was prepared for determinations which only required such accuracy; otherwise standards were run each time. No emulsions appeared at any stage of the process and interfering substances were absent.

Respiration. This was measured by the rate of oxygen absorption (QO_2) and carbon dioxide evolution (QCO_2) in a darkened conventional Warburg apparatus, at 25° C. For determination of *R.Q.* the direct method of Warburg was used (Dixon, 1943). Carbon-dioxide retention by the medium was allowed for by the method described by Umbreit, Burris, and Stauffer (1949), the pH of the cell suspension being determined electrometrically at the beginning and end of experiments, using a micro-glass electrode (Hanes, 1951).

RESULTS

The Intracellular Keto-acids of Chlorella

The existence and abundance of intracellular keto-acids when the alga was grown under reproducible, controlled conditions were first investigated. Pyruvic and α-ketoglutaric acids only were present in detectable amounts. Their identity was confirmed in two ways, viz.: (1) Comparison of the Rf.s of the DNPH derivatives with those of derivatives of known acids. (2) By conversion of the DNPH derivatives into the amino-acid analogues by catalytic hydrogenation (Fowden and Webb, 1955, following the original method of Towers *et al.* 1954). The amino-acids resulting were characterized by paper chromatography.

The extremely small amounts of keto-acids found will be noted from Table I (line 1). Although the extent of growth in the aspirator was reasonably constant from batch to batch the level of keto-acid varied rather widely. It was found that the variation in level between batches of cells could vary by as much as 50 per cent. The figures in Table I, showing the effect of changing the external conditions, are quite typical; they can be relied upon to ±8 per cent. for the batch to which they refer. Other batches would show similar trends but at differing absolute levels.

Effects of Variation of the Nitrogen Source on Level of Keto-acids

The alga was harvested, washed, and resuspended in M/15 phosphate buffer pH 6·0, and 50 ml. aliquots, each containing about 500 mg. dry wt.

cells, were transferred to sterile glass bottles of 150 ml. capacity, with sintered glass 'false bottoms' to permit forced aeration. These were arranged in an illuminated thermostat bath, and held at 25° C. Four vessels were used. To one, ammonium nitrate was added, at the same concentration as in the growth medium. Thus the medium was virtually identical with the growth medium and formed the control suspension. No nitrogen was added to the others; after 24 hours' aeration with air + 0·5 per cent. (v/v) CO_2 these cells were considered to be free of reserve nitrogen. They were referred to as 'nitrogen-starved' cells. At this point the control and one of the 'nitrogen-starved' aliquots were killed and assayed for keto-acids. To the remaining two vessels 1 g. of KNO_3 and $(NH_4)_2SO_4$ respectively, in buffer, were added. After a further 24 hours the keto-acids of these aliquots were assayed. The object of starvation was to obtain the maximum effect from the added nitrogen sources. Additional control aliquots would have been desirable but were ruled out because of shortage of material, the aliquots having to be as large as possible to get a reliable keto-acid assay. The results of this experiment are shown in Table I (lines 2, 3, 4). The effect of nitrogen starvation was to increase the concentration of both keto-acids considerably; on addition of ammonia the level dropped considerably. Nitrate had a similar but much smaller effect.

TABLE I

The Keto-acid Content of Autotrophic Chlorella

Cells were grown in mineral medium for 3–4 days, then either assayed at once ('normal'), or centrifuged, washed, and resuspended in fresh medium without nitrogen for 24 hours ('nitrogen-starved'). To the latter, KNO_3 or $(NH_4)_2SO_4$ respectively were added and the cells assayed after a further 24 hours. For other details see text.

Line	Cell condition	Keto-acid concentration (μg./g. dry wt.) Pyruvate	Keto-acid concentration (μg./g. dry wt.) α-Ketoglutarate
1.	'Normal'	49	119
2.	N-starved	134	203
3.	N-starved + KNO_3	107	114
4.	N-starved + $(NH_4)_2SO_4$	29	59

The Effect of Glucose

Under heterotrophic conditions, the alga contained considerably more keto-acid than when growth was autotrophic. Experiments demonstrating this were carried out as follows: cells grown in the normal way, on mineral medium, were harvested, washed, and resuspended in M/15 phosphate buffer pH 6·0 enriched with 5 per cent. glucose. 50 ml. aliquots (each containing about 500 mg. dry wt. cells) were transferred to sterile flasks with sintered glass false bottoms and aerated, with or without ammonium nitrate (100 mg.) for 6 hours, in darkness, at 25° C. Filtered air was used. Keto-acid assays were performed at the beginning and end of the aeration period. In this way the effect of addition of glucose, with or without nitrogen, could be compared with autotrophic cells. Aeration was for a period shorter than 24 hours to keep contamination to a minimum. It was therefore necessary to choose a period which was as short as possible consistent with obtaining nitrogen-starved cells. Six hours was considered sufficient. The results of the experiment are shown in Table II (lines 1 and 2). Comparison with the equivalent lines in Table I shows that the effect of glucose is mainly to increase the levels of keto-acids; it does not affect their relative preponderance in either normal or nitrogen-starved cells. A noticeable effect, however, is apparently to stabilize the level of α-ketoglutaric acid.

Variation of Nitrogen Supply under Heterotrophic Conditions

The procedure adopted was in many respects similar to that described in the previous section and was as follows: After growth, harvesting, and washing, the cells were suspended in M/15 phosphate buffer pH 6·0 + 5 per cent. glucose. Five 50 ml. aliquots of this suspension were taken, each aliquot containing about 500 mg. dry wt. cells. One was assayed for keto-acids at once ('normal'). The other four were aerated in the sintered glass based flasks for 6 hours, in darkness, at 25° C. Of these four only one had a nitrogen source, ammonium nitrate (100 mg.). At the end of 6 hours, the contents of this flask, and also one of the nitrogen-starved aliquots, were assayed for keto-acids. These three assays gave the levels under autotrophic, heterotrophic, and 'nitrogen-starved' heterotrophic conditions. To the remaining two 'nitrogen-starved' aliquots, 50 mg. of KNO_3 and $(NH_4)_2SO_4$ in solution in buffer were added respectively and the keto-acid level in these cells assayed after aeration for a further 30 minutes.

TABLE II

The Keto-acid Content of Heterotrophic Chlorella

For experimental details see text.

Line	Cell condition	Keto-acid concentration (μg./g. dry wt.)	
		Pyruvate	α-Ketoglutarate
1.	'Normal' . . .	140	257
2.	N-starved . . .	217	251
3.	N-starved + KNO_3 .	266	215
4.	N-starved + $(NH_4)_2SO_4$	122	200

The results are shown in Table II (lines 3 and 4). The changes in pyruvic-acid levels followed the same general pattern as the changes observed in cells incubated in the absence of glucose, although the absolute levels were two to four times as great. Changes in the level of α-ketoglutaric acid, however, were not large. The previously mentioned stabilizing effect seemed still to apply.

Once again it must be emphasized that the levels found varied widely from batch to batch of cells. The results expressed in Table II, grouped for convenience, represent a typical set of figures, to be viewed as a whole, for a particular batch of cells. Other batches of cells gave confirmatory results, but with absolute values of acid concentrations varying by up to 50–60 per cent. from those quoted.

The Gas Exchange during Starvation and Assimilation of Nitrogen

A series of determinations of the gas exchanges associated with the assimilation of nitrogen after starvation, using batches of cells exactly as in the preceding sections, were made. The results are summarized in Table III.

TABLE III

Oxygen Uptake and Carbon Dioxide Evolution of Chlorella *during Starvation and Assimilation of Nitrogen*

Flasks contained approx. 25 mg. dry wt. cells in normal culture medium, pH 6·0, without nitrogen, with or without 5 per cent. (w/v) glucose. Nitrogen substrates (30μM) added at zero time and rates of gas exchange calculated from the effect over the succeeding 30 min.

	Media without glucose	Media with glucose
	QO_2 (0–30 min.)	QO_2 (0–30 min.)
Control—without N	2·2	10·3
Ammonia added	13·8	14·9
Nitrate added	4·7	11·9
	QCO_2 (0–30 min.)	QCO_2 (0–30 min.)
Control—without N	2·5	13·1
Ammonia added	11·6	14·5
Nitrate added	11·0	17·6
	Respiratory quotients	
Control—without N	1·12	1·27
Ammonia added	0·84	0·98
Nitrate added	2·32	1·48

DISCUSSION

The extremely small amounts of pyruvic and α-ketoglutaric acids normally present in the cells of *Chlorella* would indicate that if they are intermediates in the respiratory metabolism of glucose, their turnover rate is very rapid. One can presume that when glucose was added to the cells the enzymes involved in their metabolism became saturated and a certain amount of accumulation took place.

In the present state of biochemical knowledge it is generally assumed that all, or at any rate most, of the glucose metabolized to CO_2 and water passes through the stage of pyruvic acid. A simple calculation shows that 1 g.d.wt. of cells with a QO_2 of 3–4 is producing pyruvic acid at the rate of 65μg/min.; and in the presence of glucose, with a QO_2 of 15–16, at 330μg/min. Thus the levels found in the cells under all conditions are a very minute fraction of the carbohydrate turnover.

However, the changes which occur in the levels when the alga is incubated in differing nitrogen sources are certainly significant and, under heterotrophic conditions at least, took place within 30 minutes. The gaseous exchange was also subject to rapid and considerable changes on the addition of a nitrogen source.

The increase in level of keto-acids which occurred on starvation of nitrogen was probably due to the fact that they are amino-acid precursors and the lack of available nitrogen gave rise to a slight accumulation. The accumulated acids did not form anything like an important fraction of the glucose assimilated, however, which was roughly five times the amount oxidized (Syrett, 1951; Myers, 1947).

The observation that organic acids were more abundant in cells assimilating nitrate than in those in which ammonia was the nitrogen source has been made by several authors (Clark, 1936; Wadleigh and Shive, 1939; Vickery *et al.* 1940; Bonner, 1944; and Pucher, Leavenworth, Ginter, and Vickery, 1947), although keto-acids in particular have not been investigated.

Wirth and Nord (1942), and Nord and Mull (1945) considered that the high level of pyruvate in the growth medium found when the fungus *Fusarium* was grown on nitrate was due to the inhibitory action of nitrite (produced by nitrate reduction) on pyruvic decarboxylase.

Vickery *et al.* (1940) showed wide differences in organic acid content of tobacco-plant tissues when grown on differing nitrogen sources. They studied the effect of growth under ammonium or nitrate-nitrogen conditions and suggested in a qualitative way that when a high ammonium level was supplied the organic acids were kept at a low level by reactions involving the formation of amino-acids and amides from α-ketoglutaric, oxalacetic, and pyruvic acids. This cannot, however, be a quantitative explanation since only small increases in soluble nitrogen and no increase in protein content took place. It has been suggested that nutrition with nitrate nitrogen may actively promote organic acid synthesis, possibly owing to an intracellular accumulation of mineral cations.

Warburg and Negelein (1920), and Syrett (1954, private communication) have shown that when nitrate is reduced by *Chlorella*, two CO_2 molecules are evolved per nitrate molecule reduced.

$$\begin{aligned} C_6H_{12}O_6 + 6H_2O &\longrightarrow 24H + 6CO_2 \\ 24H \quad + 3HNO_3 &\longrightarrow 3NH_3 + 18H_2O \end{aligned}$$

Therefore ratio of 'extra' CO_2 : Nitrate reduced

$$= 6/3$$
$$= 2.$$

It would seem to be probable that the necessary hydrogen atoms for nitrate reduction are derived largely from reactions coupled with decarboxylations, i.e. the Citric Acid Cycle. Table III shows that whether nitrate or ammonia was being assimilated the rate of decarboxylation was much the same, and is likely to be the maximum possible. The supply of hydrogen atoms was therefore limited and this may account for the slow rate of uptake of nitrate compared with ammonia. This reduced availability of assimilable nitrogen was in turn reflected by an accumulation of amino-acid precursors.

In the experiments with heterotrophically assimilating cells, complementary results were obtained. As was expected, the level of keto-acids rose during the period of nitrogen starvation and when ammonia was supplied the level dropped, particularly in the case of pyruvic acid. This was in spite of an increase in the rate of glucose oxidation and consequent increase in the rate of production of pyruvic acid. The *R.Q.* fell to unity, implying a transference of metabolic activity from fat production to the formation of less reduced products, probably protein. The presence of aminases and transaminases is clearly indicated. Transaminase systems have been demonstrated in the alga (Millbank, 1953).

On addition of nitrate it was expected that the diminution of keto-acids from the 'nitrogen-starved' level would not be so large, since the availability of nitrogen is more limited, for the reasons outlined above. This together with the slight increase in the rate of oxidation of glucose would be expected to cause but a slight change in the level of amino-acid precursors. These expectations were borne out in practice. The level of pyruvic acid increased slightly above that observed with nitrogen-starved cells and that of α-ketoglutaric acid fell somewhat, but the general level remained roughly constant.

Clearly, a close correlation exists between the form of nitrogen nutrition and keto-acid level in *Chlorella*, although the interrelationships between the various metabolites and intermediates is extremely complex. Demonstration of the activity of the enzymes and metabolic systems concerned will be necessary to complete the picture. Work done on this aspect will be published elsewhere.

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On *Pseudoaraucaria* Fliche emend., a Genus of Fossil Pinaceous Cones

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With Plates II and III and three Figures in the text

ABSTRACT

The type material of some fossil Pinaceous cones (from the Albian of France) originally described by Fliche in 1896 under the name *Pseudoaraucaria* is here redescribed in detail. The cones are similar in form to one from the English Lower Greensand hitherto called *Pityostrobus benstedi* (Mantell) Seward which is also redescribed here. This newly recognized group of cones differs from the cones of any of the recent members of the Pinaceae chiefly by (*a*) the massive ridge of scale tissue lying between the seeds so that these are deeply embedded in the basal part of the scale, and (*b*) the presence of a cushion of parenchyma at the chalazal end of the seed. The probable significance of these and certain other characters is discussed.

INTRODUCTION

THE generic classification of Mesozoic Pinaceous cones, like that of many other types of fossil plants, is somewhat confused. Apart from the familiar difficulty due to the almost invariably isolated state of the cones, there is also the fact that, although most of them show considerable resemblances to the cones of one or other of the living genera, they do not often fit convincingly into these genera. Most of the earlier authors (e.g. Coemans, 1866; Cornuel, 1866; Carruthers, 1866, 1869; Gardner, 1886) used either the genus *Pinus* L. (in the original very wide sense) or else Endlicher's (1847) genus *Pinites* for all cones bearing a general resemblance to the cones of living Pinaceae. As the living representatives became more precisely classified, so attempts were made to superimpose the new classification on the fossils: thus, Fliche (1896) and Berry (1911) used the genera *Pinus* (in the restricted sense), *Cedrus*, *Abietites*, and *Tsugites* for cones bearing closer resemblances to the respective living genera than to others. Stopes (1915) preferred Feistmantel's (1874) names *Pinostrobus* and *Cedrostrobus* for a similar purpose. Most recent authors, however (e.g. Boureau, 1948; Alvin, 1953), have followed Seward (1919) in returning to a wider system and using Nathorst's (1897) designation *Pityostrobus* for all fossil cones showing general Pinaceous characters. This system, although simple and convenient at the present time while so little is known about the range of form and structure amongst Mesozoic Pinaceae, should be regarded as provisional. In due course, as detailed descriptions of more species are published, natural generic groupings will probably become

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recognized. Some of these may prove to be closely similar to certain recent genera, but others, of which the one forming the subject of this present paper is probably the first example, will be distinctly different.

The genus *Pseudoaraucaria* Fliche (1896) was erected for some fossil cones (from the Albian of France) believed to occupy a systematic position between the Pinaceae and the Araucariaceae: the structure seemed to be similar to that of an *Araucaria* cone, but each scale carried two seeds instead of one. With the kind permission of the Director of the École Nationale de Géologie Appliquée, Nancy, where the type material of *Pseudoaraucaria* was deposited, I was able to borrow all that remained of this material for the purpose of comparing it with a certain species of cone from the Wealden of Belgium. Upon examination it became evident that Fliche's cones were essentially Pinaceous, and that they had the same peculiar structure as the Belgian cone. This latter is therefore being described under the name *Pseudoaraucaria heeri* (Coemans). (Alvin, *in press.*)

Fliche's descriptions were found to be not only inadequate in view of the excellent state of preservation of the material, but also misleading owing to his misinterpretation of the form of the scale. It is the aim of this present paper, therefore, to give a detailed redescription of the type material from France, and also of the hitherto somewhat problematical British species *Pseudoaraucaria benstedi* (Mantell) comb. nov. which appears to belong to the same group.

Material and Methods

The French material is on the whole extremely well preserved, and has lent itself well to the 'peel' technique. The petrifying mineral is mainly calcium carbonate, but the outer part of the cone of *Ps. loppineti* contains a considerable amount of an opaque, granular substance which is probably iron pyrites. Most of the fragments from which sections were prepared were small, and had to be supported either in a plastic substance or in plaster, but apart from this the material presented no difficulty. Macerations (in Schultz's Fluid followed by alkali) after demineralization in strong hydrochloric acid were carried out in the hope of finding pollen grains, but they yielded nothing but the tough megaspore membranes.

The cone of *Ps. benstedi* is silicified and suffers from the disadvantage of having almost colourless cell walls. Several 'peel' sections have been made, and these, together with the four rock sections made by Stopes (1915), have been adequate to show the structure of the cone.

Emended Generic Diagnosis

The following emended generic diagnosis is suggested:

Pseudoaraucaria Fliche emend.

Pinaceous cone, ellipsoidal or shortly cylindrical in shape.

Bract short, simple, free from the ovuliferous scale from the position of the bend in the latter or below.

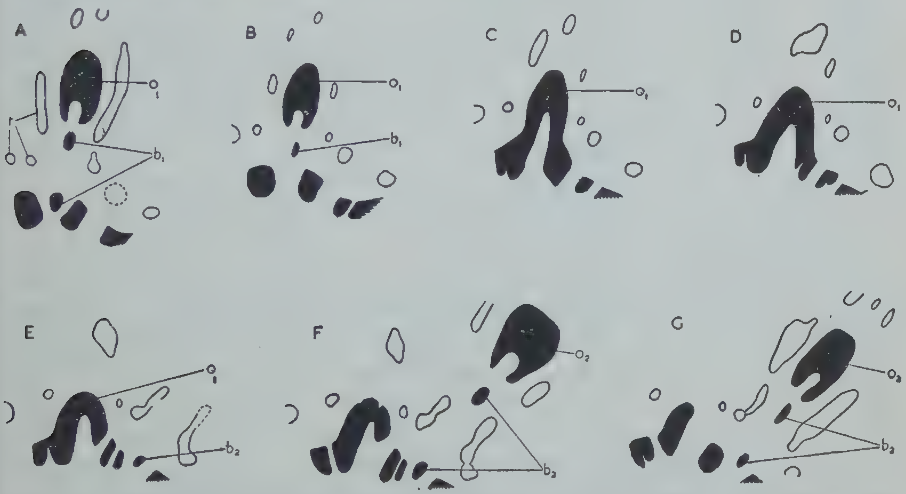
Ovuliferous scale sharply bent; the lower part set at about a right angle to the axis and carrying two seeds separated by a prominent ridge of scale tissue; the upper part laminoid, set at almost a right angle to the lower. Resin canals in the lower part of the scale distributed entirely adaxially to the vascular system, except for two which enter the bract.

Seed winged, pointed at the micropylar end, broad at the chalazal end; sclerotesta smooth except often at the base where it gives place to a soft tissue (often partially or completely broken down) continuous with the base of the wing.

DESCRIPTIONS OF SPECIES

Pseudoaraucaria loppineti Fliche

Pseudo-Araucaria loppineti Fliche, 1896, p. 189; Pl. VI, Figs. 3–5.
Pseudo-Araucaria lamberti Fliche, 1896, p. 193; Pl. VII, Fig. 2.



TEXT-FIG. 1. *Pseudoaraucaria loppineti.* An acropetal series of drawings made from transverse sections of the cone axis, showing the mode of origin of the vascular traces of the cone appendages. b_1 and b_2 are bract traces; o_1 and o_2 are the traces of the corresponding ovuliferous scales; *r*, resin canals. Fig. A is from slide C 17; Fig. B, C 15; Fig. C, C 12; Fig. D, C 11; Fig. E, C 10; Fig. F, C 9; Fig. G, C 8. All ×5.

The only material of Fliche's *Ps. lamberti* in the Nancy collection consists of two incomplete scales. I have sectioned these in both transverse and longitudinal planes, and have compared them with the scale of *Ps. loppineti.* I have discovered no essential differences between these 'species' and have therefore combined them here under the name having priority.

Although Fliche recorded three specimens of *Ps. loppineti,* there is apparently now only one at Nancy; the fate of the other two is unknown. The existing specimen, upon which the following description is based, may be taken as the type-specimen.

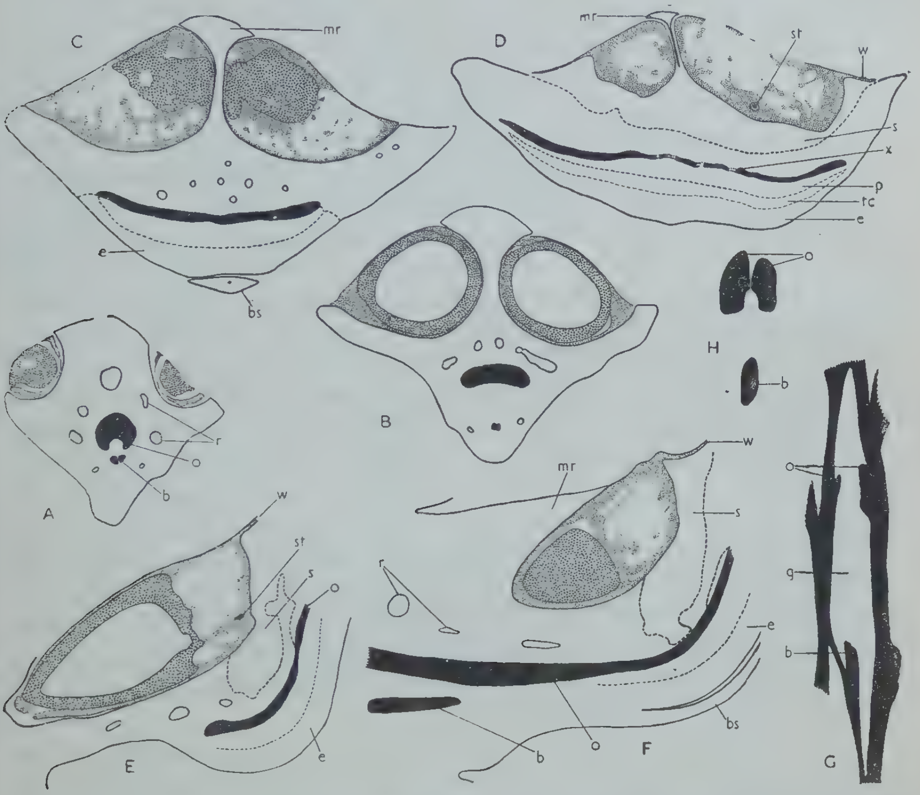
The specimen had at some time been cut in a median longitudinal plane, and had subsequently either broken up or else been dissected. It has been possible to piece together one-half of the cone (Pl. II, Figs. 1 and 2), and some of the other fragments (mainly scale bases) have been used in the preparation of thin sections, and for macerations.

The cone is ellipsoidal in shape. It has an over-all length of 5·4 cm. and a breadth of 3·1 cm. These dimensions, however, cannot be taken as those of the original cone, because the upper portions of the scales are missing, probably having been either worn away or broken off before the cone became preserved. The outer surface of the fossil shows rhomboidal areas representing the abraded or broken ends of the scales, and bears irregular ridges where the harder tissues have been exposed (Pl. II, Fig. 1). At the base of the specimen there is a protuberance that looks like a peduncle, but it is by no means certain that this is what it was; for one thing, it is a good deal narrower than the lowest part of the cone axis, and is not quite in alignment with it (Pl. II, Fig. 2); and further, it contains no preserved tissues, but consists wholly of mineral matter.

The cone scale, as is usual in the conifers, is a compound organ. The bract, in the axil of which, fundamentally, is borne the ovuliferous scale (or 'seed-scale complex', to follow Florin's (1951) terminology), is fused to the latter for part of its length. It becomes free at about the point where the ovuliferous scale bends upwards (Text-fig. 2 F). The tongue-like free part of the bract is about 5 mm. long by 2 mm. broad. The part which is fused to the ovuliferous scale broadens towards the cone axis.

The basal part of the scale is set at about a right angle to the axis, although near the apex of the cone this angle becomes more acute, and towards the base, more obtuse. At its attachment to the axis the scale is narrow (Text-fig. 2 A), but farther out it widens. A vertical section through this part of the scale (i.e. tangential to the cone) is nearly rhomboidal in outline, about 8 mm. high. There are two seeds which lie along the length of this horizontal part of the scale, and which are separated from each other by a massive ridge of scale tissue (the interseminal ridge); the seeds are thus embedded in the scale. As the distal part of the scale is missing, it is not possible to say how the ridge terminated; it merely fades out just beyond the plane of section in Text-fig. 2 D. It probably joined up with the erect part of the scale beyond the seeds. Text-fig. 2 D shows the ridge entirely free from the rest of the scale below, but this is probably due only to the rather poor preservation of the tissues in this region. (The approximate limit of the preserved tissues is indicated in the drawings by a thick broken line.) Of the distal upturned portion of the scale, only the very lowest part is present (Text-fig. 2 F; Pl. II, Fig. 5). It was probably set at about 120° to the horizontal seed-bearing portion. The angle is rather less sharp than in *Pseudoaraucaria heeri* where it is almost exactly a right angle (Alvin, *in press*). No accurate estimate can be given of the length of the upturned part, but its vascular supply is very considerable (Text-fig. 2 D). If the scale proportions were the same as in the somewhat smaller *Ps. heeri*, then the upper part was probably about 1·5–2 cm. long. In *Ps. heeri*, the scale bore a conspicuous terminal appendage or umbo, like that in some of the recent species of *Pinus*, but it is of course quite impossible to say at present whether there was a similar appendage in *Ps. loppineti.*

The seed is fairly large, being 7–8 mm. long and 3–4 mm. in diameter. In transverse section (Pl. II, Fig. 3) it is ovate in outline, the more pointed edge being outwards and lying close to the outer margin of the scale. There is some indication (Text-fig. 2 D, E, and F) that the seed bore a wing of the type found in the majority of Pinaceae.



TEXT-FIG. 2. *Pseudoaraucaria loppineti.* Figs. A–D, a series of vertical sections (tangential to the cone) through the base of the scale. Figs. E and F, longitudinal sections through the base of the scale. Fig. G, part of the axial vascular system in tangential longitudinal section, showing the mode of origin of the vascular traces to a cone appendage. Fig. H, the vascular supply as seen in tangential section through the cortex of the axis. *bs*, bract; *b*, bract trace; *o*, ovuliferous scale trace; *g*, 'leaf-gap'; *st*, vascular trace of the seed; *mr*, interseminal ridge; *w*, seed-wing; *e*, outermost abaxial tissue of narrow, elongated cells (shown in Text-fig. 3 A); *tc*, layer of thick-walled cells (shown in Text-fig. 3 C); *p*, layer of large-celled tissue (shown in Text-fig. 3 B); *r*, resin canals; *s*, space, probably where tissues have broken down; *x*, point at which the seed trace joins the scale strand. Fine stippling indicates the outer layer of the integument; the sclerotesta is coarsely stippled. Fig. A is drawn from slide B 1; Fig. B, B 5; Fig. C, B 9; Fig. D, B 14; Fig. E, A 5; Fig. F, A 1; Fig. G, A 5; Fig. H, A 7. All except Figs. G and H ×5. Figs. G and H ×7½.

Structure of the axis. In the centre of the axis is a homogeneous pith which, except where it narrows towards the apex, is about 4·5 mm. in diameter. It is composed of more or less isodiametric cells with conspicuously pitted walls.

The vascular cylinder (about 1 mm. thick) is greatly dissected by gaps associated with the departure of scale traces. Text-fig. 1 shows in a series of drawings made from sections cut at successively higher levels, the mode of departure of the traces. Although usually the traces pass through the cortex almost horizontally, in the lower part of the cone from which region these sections were prepared they take a slightly downward course. Hence, the small bract trace (b_1) in Fig. A is seen cut twice, and the ovuliferous scale trace (o_1) is seen in the cortex in the same figure, whilst at a somewhat higher level (Fig. C) this same trace is seen to join the axial cylinder. The bract trace arises not at the bottom of the associated gap (the 'leaf-gap') as in other fossil Pinaceous cones I have examined (Alvin, 1953, and *in press*), and as in most living Pinaceae (Radais, 1894), but it arises on one side some distance above the bottom (Text-fig. 1, D, E, and F; Text-fig. 2 G). The trace to the ovuliferous scale consists of two strands arising one from each side of the gap (Text-fig. 1 C, *o*). These strands soon fuse to give a single horseshoe-shaped mass similar to that found in most of the other Pinaceae. Sometimes, especially near the apex of the cone, the bract and scale traces tend to fuse together to form a cylinder, as they do in some living species of *Pinus*.

The vascular tissue, both of the axial cylinder and also of the traces, consists chiefly of very thick-walled tracheids arranged in radial files. Some of the tracheids of the axis show uniseriate bordered pits on the radial walls. Besides the broad 'leaf-gaps' there are uniseriate medullary rays. It is not clear whether ray tracheids are present, nor is there any clear indication of the presence of resin canals in the wood.

The axial cortex, as is usual in the Pinaceae, contains a system of resin canals. The vertically running canals number about 20. Radais (1894) showed that this canal number tended to be related to the phyllotaxy and was usually one of the series 1, 2, 3, 5, 8, . . ., 21 being a number that occurs frequently amongst living and fossil members of the family; the number in this fossil may therefore be 21. The vertical canals ramify, and branches pass out into the scale bases (Text-fig. 1 F and G). It is probable that branches from two axial canals supply the scale. Nearly all resin canals contain a yellowish substance which is probably fossil resin, and opaque granules like those found plentifully elsewhere in the fossil are also usually present. The epithelial layer is seldom preserved.

The cortical cells resemble those of the pith; but in the outer cortex they merge into thicker-walled, radially elongated cells continuing into the scale bases.

Structure of the scale. On entry into the combined cone appendage, the strongly curved scale trace runs closely above the small bract trace (Text-fig. 2 A). The bract strand at this level appears to be divided into two by a narrow ray of dark parenchyma. This division occurs also in a number of recent members of the family (Radais, 1894). I have not observed it, however, in

Pseudoaraucaria heeri, possibly because the preservation was not sufficiently good (Alvin, *in press*).

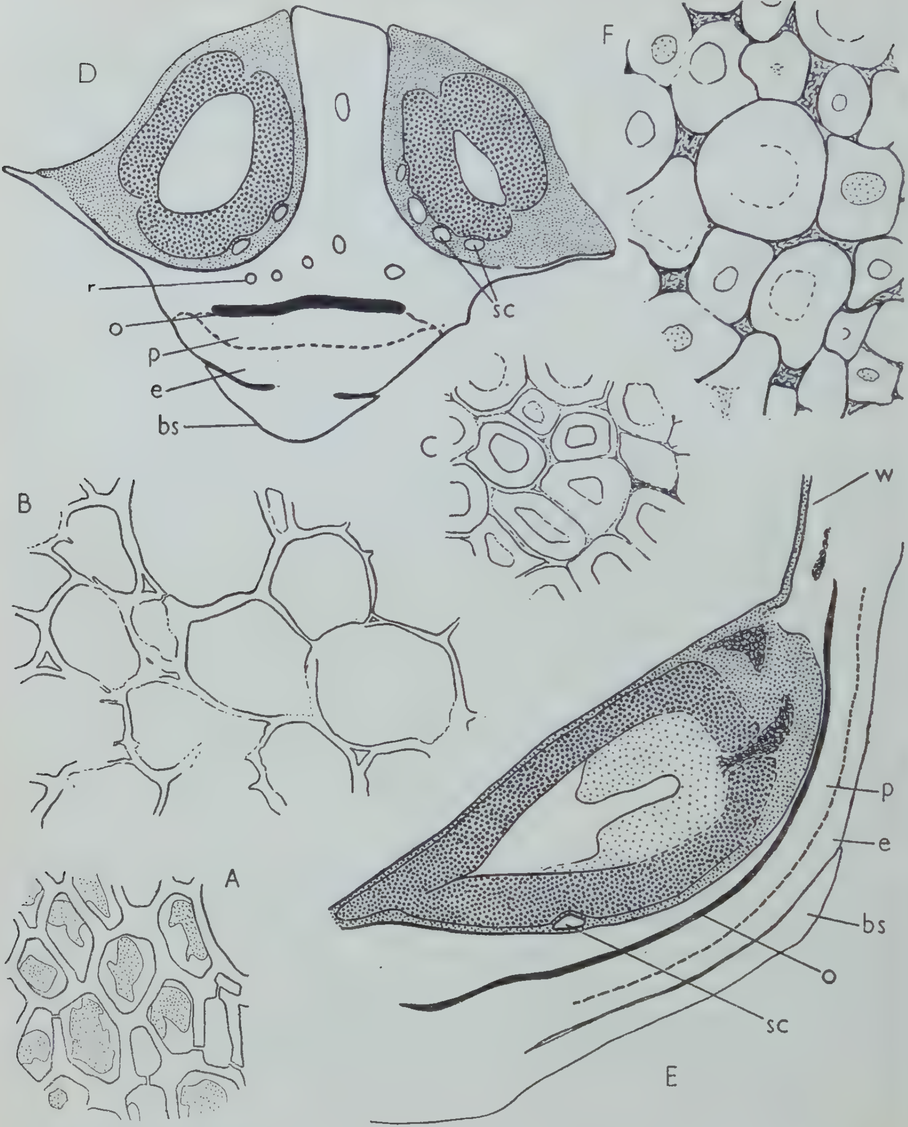
Farther outwards (Text-fig. 2 B, C, and D) the two strands in the scale base move farther apart. The bract trace gradually diminishes, and the dividing ray disappears before the trace enters the free part of the bract (Text-fig. 2C); soon after this it fades out. The ovuliferous scale trace gradually loses its curved form and broadens markedly (Text-fig. 2 B and C). It is doubtful whether this broad band ever breaks up into smaller strands in the course of the preserved part of the scale. The breaks shown in Text-fig. 2 D are probably not real, since in the next section of the series some of them have disappeared: they are probably due solely to the rather poor preservation in this region of the scale.

It is almost certain that seed traces were given off from the scale strand at somewhere about the level as the section in Text-fig. 2 D. In this section there is a small dark spot (*st*) in the chalazal part of the right-hand seed which probably represents a small vascular bundle. In the next section (outwards) this has vanished, and a small protuberance has appeared on the scale strand just beneath the seed (at the point *x* in Text-fig. 2 D) as if a small vascular strand had just joined it. The appearance of the seed trace in longitudinal section as it passes through the soft chalazal tissue is shown in Figs. 5 and 6, Pl. II. There is some indication (Fig. 6) that it contained scalariform elements.

As in the axis, the vascular strands in the scale consist almost entirely of xylem, the elements of which are arranged in radial rows. The phloem is not preserved, but it may reasonably be assumed that this tissue lay along the lower side of the xylem in the bract trace, and on the upper side in the scale trace, where there is often a thin layer of black material which may represent the phloem.

The secretory system of the scale at the extreme base (Text-fig. 2 A) consists of a ring of canals distributed round the vascular strands. The four large upper ones in this ring belong to the ovuliferous scale, and the two lower ones, which do not divide further, accompany the bract trace for a distance before eventually fading out. The ovuliferous scale canals always remain above the vascular system throughout the preserved part of the scale. We do not know what happens in the distal part, but in *Pseudoaraucaria heeri*, as well as in several other fossil and living Pinaceae, the canals become more scattered in the upper region.

The basal part of the scale is largely sclerotic. The interseminal ridge and the tissues lying beneath the seeds, as well as much of the bracteal part, are composed of very thick-walled, pitted cells (Pl. II, Figs. 3 and 4). The cells are usually round in transverse section, but less regular and often more or less elongated in the longitudinal direction. Immediately beneath the vascular strand there is a rather dark parenchyma. This is at first very small in extent, but as the vascular band broadens, so this tissue increases. Just before the scale bends upwards the abaxial tissue becomes differentiated into first two and then three layers (Text-fig. 2 C–F). The outermost (*e*) is fairly thick and



TEXT-FIG. 3. A–C, *Pseudoaraucaria loppineti*, transverse sections of the three types of tissue lying abaxial to the vascular strand in the scale; A, the outermost; B, the innermost; C, the middle layer. D–F, *Ps. benstedi.* D, vertical section (tangential to the cone) through the base of the scale. E, radial longitudinal section through a scale. F, cells from the outer abaxial tissue. *bs*, bract; *o*, trace of ovuliferous scale; *r*, resin canals; *sc*, supposed secretory cavities in the testa; *w*, seed-wing; *p*, parenchyma lying immediately abaxial to the vascular strand; *e*, abaxial thick-walled cells shown in Fig. F. The outer layer of the testa is finely stippled, the sclerotesta is coarsely stippled. D and F are drawn from B.M. slide no. 39107 c; E from 39107 a. Fig. A is from slide B 11, Figs. B and C from B 7. D and E are magnified × 10, the rest × 40.

composed of narrow, elongated, relatively thin-walled cells with dark contents (Text-fig. 3 A). The middle layer is thinner, and made up of thick-walled fibres (Text-fig. 3 C). The innermost tissue is a large-celled parenchyma (Text-fig. 3 B). The three tissues tend to intergrade (Pl. III, Fig. 5).

Structure of the seed. The most striking feature of the seed is the presence of a massive pad of sterile tissue situated at the chalazal end and occupying about one-third of the total length of the seed (Text-fig. 2 E and F). This probably consisted of a homogeneous delicate parenchyma, but the tissue is never well preserved, and contains much opaque mineral matter. The centre is often occupied by a large, irregular space. Fliche (1896) believed this was a resin cavity, but there seems to me nothing to suggest that it ever contained resin: there is no epithelial layer, nor is there any fossil resin such as is found in the resin canals. Running through the chalazal tissue is the single vascular trace; this seems to end at a point in the base of the sclerotesta (Pl. II, Fig. 5).

The integument (Pl. II, Fig. 4) is differentiated into the usual three layers, although the innermost is represented only by a thin, dark band inside the prominent middle layer, the sclerotesta. This latter is massive, being about 6–8 cells (0·3 mm.) thick, except at the corners beneath the two ribs of the outermost layer where it is thinner. The surface of the sclerotesta is generally smooth, but often somewhat irregular at the base (Text-fig. 2 E). It is surprising that the stony tissue making up this layer is not better preserved, but it is often difficult to pick out individual cells; however, they appear to be typical stone-cells with extremely thick walls and abundant pits, and are very compactly arranged without intercellular spaces. The outer fleshy layer of the integument is directly continuous with the parenchymatous chalazal tissue. Most of this layer is thin, consisting of about three rows of small, delicate, rather flattened cells; but along the upper and lower edges of the seed it is very much thicker, so as to form two longitudinal ribs. (In Text-fig. 2 B–F the lightly stippled parts represent this outer integumental layer, the broader parts being the ribs.) The ribs, the lower one of which is stouter than the upper, have a core of stone-cells apparently similar to those of the sclerotesta; but thin-walled cells (sometimes broken down to give a space) separate the core from the sclerotesta, and there are also thin-walled cells outside the core (Pl. II, Fig. 4, top right-hand corner).

I have seen no resin cavities anywhere in the testa. There are occasional small spaces between the outer layer and the sclerotesta, but these show no sign of having been of a secretory nature.

The integumental structure is remarkably similar to that described by Quisumbing (1925) in *Pinus banksiana*. In all species of *Pinus*, although in no other genus of the Pinaceae, the integument is clearly two-ribbed, and its structure is probably similar to that in *Pinus banksiana*. According to Quisumbing, the cells of the sclerotic core in the rib have round lumina with smooth, unpitted walls, and are therefore of a different character from the stone-cells of the sclerotesta. The preservation in *Pseudoaraucaria* is hardly good enough to allow one to recognize a similar distinction there.

The seed contents are sometimes almost perfectly preserved. The seeds were fully grown and mature, for they frequently contain a well-developed endosperm and embryo. The endosperm, which is of course the ripe tissue of the female gametophyte, is present within the megaspore membrane. This latter is thick, finely papillate, and of the typical gymnosperm type (Thomson, 1905). Outside the membrane is a thin layer of dark material which probably represents the remains of the nucellus together with the innermost layer of the integument.

Unfortunately it has not been possible to prepare good thin sections of the endosperm and embryo, because the tissues, although calcified, are very delicate, and do not stand up well to etching, even in very dilute acid. The structure of the seed contents has therefore been studied by examining wetted surfaces by reflected light: the photograph on Pl. II, Fig. 7 was taken by this means. The endosperm is composed of uniform, rather rounded cells with thin walls. There is a tendency for each cell to contain a single dark spot, but whether this represents the shrunken cell contents or the nucleus, or whether it is only a mineral deposit is not known. There are clear indications of how some of the cells have divided during the course of development.

The embryo is of the Pinaceous type. Its total length is about 3 mm. The radicle takes up about half this length and is approximately 0·5 mm. in diameter; it appears to contain a dark central strand. There were seven cotyledons in the two embryos that I examined in transverse section. The cotyledons are arranged in a circle which has a maximum diameter of about 1 mm. Each one is sector-shaped in section, and contains a single dark strand in the centre which presumably represents a vascular bundle. Towards the inside of the cotyledon the cells are about 20 μ in diameter, and again, like the cells of the endosperm, they often contain dark spots. Towards the outside there are one or two layers of much smaller cells. No resin ducts are visible in the embryo.

Discussion

Pseudoaraucaria loppineti is very similar to the somewhat older *Ps. heeri* from the Wealden of Belgium (Alvin, *in press*). The main differences are as follows:

1. *Ps. loppineti* is generally rather larger.
2. The scale is less sharply bent than in *Ps. heeri*.
3. There are some thick-walled fibres in the abaxial tissues of the scale which are absent in *Ps. heeri*.
4. The vascular trace of the bract arises from the side of the 'leaf-gap' rather than from the bottom.
5. Integumental ribs were not evident in *Ps. heeri*, although the integument was sometimes thicker at the angles than at the sides of the seed.
6. The chalazal cushion of parenchyma was apparently small in *Ps. heeri*.
7. No seed trace was demonstrated in *Ps. heeri*.

With regard to the last three points, it should be emphasized that the preservation of the seed in *Ps. heeri* was not as good as in *Ps. loppineti*, and it is consequently difficult to be sure of these differences. Besides the generally poorer preservation, the thickness of the integument, and the relative thicknesses of the sclerotesta and outer layer varied somewhat from one specimen to another in *Ps. heeri*; in some specimens the outer layer was definitely thicker in the corners so as to form ribs perhaps like those in *Ps. loppineti*. Poor preservation might account for the virtual disappearance of the soft chalazal tissue in *Ps. heeri*, and also for the apparent absence of a seed trace.

The other distinguishing characters, however, together with the difference in age, give sufficient grounds for regarding the two species as being quite distinct.

EMENDED SPECIFIC DIAGNOSIS

Pseudoaraucaria loppineti Fliche, emend.

Cone ellipsoidal; incomplete but probably originally 5–6 cm. long and about 3·5 cm. in diameter.

Bract (free part) 5 mm. long, 2 mm. broad.

Scale bent at an angle of about 120°; lower part of typical scale 13 mm. long, 12 mm. broad, 8 mm. high.

Seed 7–8 mm. long, 3–4 mm. in diameter; with a massive parenchymatous chalazal part 2–2·5 mm. long; sclerotesta 0·3 mm. thick; outer layer of testa with two thick longitudinal ribs.

Embryo about 3 mm. long, with 7 cotyledons.

Vascular trace to the bract leaving the axial cylinder from one side of the 'leaf-gap' near the bottom. Trace to the ovuliferous scale consisting of two strands, one from each side of the gap, but fusing in the cortex to form a horseshoe-shaped strand.

HORIZON: Albian.
LOCALITIES: Vaubécourt (Meuse), France.
Grandpré (Ardennes), France.

Pseudoaraucaria major Fliche

Pseudo-Araucaria major Fliche, 1896, p. 191; Pl. VII, Fig. 1.

Only two isolated scale bases belonging to this species remain in the Nancy collection, in spite of the fact that Fliche recorded two cones. Both scale fragments have been examined and sectioned; they proved to be rather similar in form and structure to the scale of *Ps. loppineti*, but could be distinguished on the following characters:

1. The size is greater: the length of the scale base is 16 mm., and the greatest breadth is 16 mm.; the height is about 8 mm., but since nearly all the tissue lying below the vascular band of the ovuliferous scale is missing, the original height was probably about 1 cm.

2. The seed is considerably larger, being about 12 mm. long. The seed

cavity (which is empty) is 5 mm. long. The chalazal cushion, the centre of which is broken down to form another cavity, is about 4 mm. long.

3. The sclerotesta is only about half as thick as in *Ps. loppineti*, being about 0·16 mm.

4. The horseshoe-shaped scale trace has a core of thick-walled cells (Pl. II, Fig. 7). Unfortunately, owing to the rather poor preservation, the detailed structure of this sclerenchyma could not be observed, but it appeared to be fibrous.

Although these differences indicate that *Ps. major* is distinct from *Ps. loppineti*, the two species are almost certainly closely related.

The characters pertaining to the whole cone in the following emended diagnosis are taken from Fliche's original diagnosis.

EMENDED SPECIFIC DIAGNOSIS

Pseudoaraucaria major Fliche, emend.

Cone sub-cylindrical, 4·9 cm. (at least, because of abrasion) in diameter.

Scale bent, the lower part about 16 mm. long, 16 mm. broad, 10 mm. high.

Seed 12 mm. long, with a massive sterile chalazal part about 4 mm. long; sclerotesta 0·16 mm. thick; outer layer of testa with two thick longitudinally running ribs.

Vascular trace of the ovuliferous scale horseshoe-shaped, the medulla of this trace enclosing a core of sclerenchyma.

HORIZON: Albian.

LOCALITY: Les Islettes (Meuse), France.

Pseudoaraucaria benstedi (Mantell) comb. nov.

Abies benstedi, Mantell, 1843, p. 34. 1844, p. 166. 1846, p. 52; Pl. II, Figs. 2, 2 *b*, 2 *c*.
Pinites benstedi, Endlicher, 1847, p. 19.
Abietites benstedi, Goeppert, 1850, p. 207.
Pinites benstedi, Carruthers, 1866, p. 541. 1867, p. 12.
Cedrus benstedi, Schimper, 1870, p. 300.
Abietites benstedi, Gardner, 1886, p. 246.
Pinostrobus benstedi, Stopes, 1915, p. 130; Pl. X, Fig. 1; Pl. XI, Figs. 1 and 2; Text-figs. 32 and 33.
Pityostrobus benstedi, Seward, 1919, p. 386; Fig. 780.

This interesting cone, of which only a single specimen is known, comes from the Lower Greensand (Aptian) of England. As is suggested by the several taxonomic changes that the species has undergone, its affinities within the Pinaceae have always been uncertain. Until Stopes (1915) attempted an anatomical study, hardly more than the gross form of the cone had been described; and although Stopes's work revealed certain interesting features it unfortunately suffered from a misinterpretation of the form of the scale base, with the result that the bract was believed to be relatively massive.

As in the specimen of *Ps. loppineti*, the ends of the scales are missing, so that it is not possible to give an accurate description of the external features. The cone was probably a good deal smaller than *Ps. loppineti*, since the present length of the specimen is only 4 cm., and its diameter is only 2·8 cm. It is more or less barrel-shaped, and the apex is slightly depressed as in the recent *Abies* and *Cedrus* and in certain fossil cones including *Pseudoaraucaria heeri* (Alvin, *in press*).

The individual scales are also smaller, having a maximum breadth of about 1 cm. and thickness of about 7 mm. The lower part of the scale merges more gradually into the upper part than it does in the other species of *Pseudoaraucaria*, and the two parts make an angle of as much as about 135° with each other (Text-fig. 3 E). The vertical section through the base of the scale shows two differences from that in *Ps. loppineti*; firstly, the interseminal ridge is less markedly broadened at its crest, so that it scarcely overarches the seeds (Pl. III, Fig. 3; Text-fig. 3 D), and secondly, the lateral parts of the scale lying beneath the seeds become extremely thin (Text-fig. 3 D), and it is not unlikely that the seeds may actually overlap the edges of the scale.

The anatomy of the cone is similar to that of *Ps. loppineti*. The axis is composed largely of fairly thin-walled cells, but the scale base is made up mainly of very thick-walled sclerenchyma. The tissues lying abaxial to the vascular strand in the more distal part of the scale base and in the bottom of the upturned part are differentiated into two zones (Text-fig. 3 E; Pl. III, Fig. 3). The inner is composed of a rather large-celled parenchyma, and the outer (the dark zone) is of smaller cells, some of which show dark contents (Text-fig. 3 F). These cells are considerably larger than the cells in the same position in *Ps. loppineti* (cf. Text-fig. 3 A), and they are probably structurally different. It is difficult to decide, owing to the almost colourless nature of the cell walls, whether these cells have thin or thick walls, or even whether the tissue is mixed. Some cells contain a dark centre which may represent either the completely filled lumen or only the cell contents shrunken away from the thin walls; other cells contain only a faint circle (indicated in Text-fig. 3 F by a broken line). It seems to me most likely that the cells were thick-walled and fibre-like, and similar to the cells in the abaxial part of the scale base in nearly all recent Pinaceae (Radais, 1894).

The vascular system is similar in form, but generally less well developed than in *Ps. loppineti*. The axial cylinder is only about 0·3 mm. thick, and the scale traces are small. As before, the vascular tissue is represented by little else but the secondary xylem, and this appears to be similar in structure to that in *Ps. loppineti*; the uniseriate pitting of the tracheids is shown on Pl. III, Fig. 2. I have not been able to ascertain the mode of origin of the bract trace.

The seed is about 6 mm. long and 3 mm. broad. At the chalazal end there is a parenchymatous cushion (Text-fig. 3 E), but this is much less extensive and a different shape from that in *Ps. loppineti* and *Ps. major*. It appears to be composed of soft parenchyma which has sometimes broken down to give dark patches. I have seen no definite vascular trace, but there is a break in the sclerotesta through which a dark, apparently amorphous material passes (Text-fig. 3 E): it is possible that this may represent the remains of the seed trace. The most important structural distinction between this seed and that of any other species of the genus is the fact that what are almost certainly secretory cavities are present in the outer layer of the testa. These, which can be seen clearly in Fig. 1, Pl. III and in Text-fig. 3 D, are about 0·2 mm. across, and occupy slight hollows in the sclerotesta. They are lined by several layers of flattened cells, and appear similar to the resin cavities in the seed-coat in certain recent genera of Pinaceae. I have, however, seen no sign of fossil resin such as is usually visible in the ordinary resin ducts, in the seed cavities in the fossil. It is possible, although perhaps unlikely, that the cavities contained a mucilaginous substance which has completely disappeared.

Details of integument structure in *Ps. benstedi* are difficult to make out because of the rather poor state of preservation. The outer layer is probably thicker than in *Ps. loppineti*, and is especially massive in the corners, where there appear to be sclerotic ribs. Seed contents are not preserved.

Discussion

The most important character distinguishing this species from the other species of *Pseudoaraucaria* is the presence of secretory cavities in the testa. Amongst living Pinaceae, resin cavities are invariably found in the seed-coat in *Abies*, *Tsuga*, *Cedrus*, and *Keteleeria*, and are always absent in *Pinus*, *Picea*, *Larix*, and *Pseudotsuga*. The character thus seems to be of some taxonomic significance, especially as these groups of genera were recognized by Jeffrey (1905) as sub-families (Abieteae and Pineae) which could be distinguished on several characters. However, it is by no means certain that these form truly natural groups in which all members are more closely related to one another than to others; *Pinus* and *Pseudotsuga*, for example, seem to have much less in common than *Pseudotsuga* and *Tsuga*, not only in the form and structure of their cones, but also in their vegetative organization.

The only other difference which is not merely one of degree, between *Ps. benstedi* and the other species, is the fact that the cells of the abaxial tissue in the scale are larger and probably thicker-walled. This, however, although a useful point of distinction at the specific level, does not provide grounds for a generic separation; it is not as great a structural difference, for example, as that between the scales of the two living species of Keteleeria (Alvin, 1953).

The classification of this species in the genus *Pseudoaraucaria* seems justifiable at the present time because it has more in common with the other species of this genus than with any other known type of Pinaceous cone. Among the most striking points of similarity are: (*a*) the presence of a massive ridge of tissue between the seeds; (*b*) a similar integument structure; (*c*) the presence of a parenchymatous cushion at the base of the seed; (*d*) the same general shape and proportions of the cone and scales.

EMENDED SPECIFIC DIAGNOSIS

Pseudoaraucaria benstedi (Mantell) comb. nov.

Cone barrel-shaped; apex depressed; about 4 cm. long, 2·8 cm. in diameter.

Scale typically bent at an angle of about 135°; lower part about 8 mm. long, 7 mm. broad, 5 mm. high.

Seed about 7 mm. long, including the chalazal cushion about 1·5 mm. long; sclerotesta 0·2–0·4 mm. thick; outer part of the integument with two longitudinal ribs, and containing secretory cavities.

Vascular trace to the bract simple; trace to the ovuliferous scale horseshoe-shaped, derived from two strands arising from the sides of the 'leaf-gap' in the axial cylinder.

HORIZON: Kentish Rag, Lower Greensand (Aptian).

LOCALITY: Iguanodon Quarry, near Maidstone, England.

GENERAL DISCUSSION

An extensive comparative discussion on the species *Pseudoaraucaria heeri* from the Wealden of Belgium is being published elsewhere (Alvin, *in press*), and very little more can profitably be said at present on the possible relationships between *Pseudoaraucaria* and other fossil and living members of the Pinaceae. However, it seems desirable to discuss briefly the significance of those features which are new in the species described here, and also to consider the wider phylogenetic significance of certain peculiar characters of the genus as a whole.

The most conspicuous feature of the genus is the massive interseminal ridge. Although a ridge is present in this position in all members of the family, nowhere is it as large as in *Pseudoaraucaria*. The morphological nature of the ridge is obscure. It is essentially proximal, fading out just above the seeds, and it never contains vascular tissue, although it may be richly supplied with resin ducts. It is just conceivable that it may represent the adaxial member of a proximal pair of sterile scales (that is, the decussate pair below the predominating pair) in the seed-scale complex (see Florin, 1951). However, there is no palaeobotanical or anatomical evidence in support of this theory, and it is probably safer to regard the ridge as either an outgrowth from the scale or else merely the result of the seeds having become more or less embedded in the tissues of the scale. Thus in *Pseudoaraucaria* where the seeds are deeply embedded the ridge is large, and in *Pinus* where they are scarcely at all sunken it is extremely small.

In *Pseudoaraucaria heeri* a large terminal umbo is a conspicuous feature of the scale. Although *Pseudoaraucaria* differs in several important ways from *Pinus* (Alvin, *in press*), there is no reason to suppose that the umbo is morphologically different from the similar appendage found in the *Diploxylon* section of *Pinus*. This appendage Florin (1951) believes represents the adaxial member of a second (distal) pair of sterile scales in the seed-scale complex. It is unfortunate that the upper parts of the scales should be missing in the three species of *Pseudoaraucaria* described here: it may be that the presence of an umbo was a generic character.

The seed structure, which, as has already been suggested, may be fundamentally the same in all four known species of *Pseudoaraucaria*, is highly distinctive, chiefly because of the usually massive pad of soft tissue present at the chalazal end. It seems unjustifiable to regard this cushion as being merely the thickened basal part of the seed-wing, although the thin wing proper is continuous with it at its upper distal edge, because firstly, it does not taper off gradually into the wing, but leads into it abruptly, and secondly, as has been shown in *Ps. loppineti*, the vascular trace of the seed passes longitudinally through it. Amongst living Pinaceae there is no structure which approaches this chalazal cushion in massiveness, although in some (e.g. *Pinus* spp.) the base of the wing is somewhat thickened. Amongst other conifers, some members of the Taxodiaceae (e.g. *Cunninghamia*) possess short, pad-like protuberances on the upper surface of the scale, upon which the seeds are carried. These, however, as is suggested by the organization of the vascular system (Radais, 1894), and confirmed by Florin's (1944, 1951) comparative studies, represent the whole sterile part of the seed-scale complex, and cannot therefore be morphologically equivalent to the chalazal cushions in *Pseudoaraucaria*. It is perhaps more useful to compare the fossil with some of the older (Palaeozoic and early Mesozoic) conifers in which the seed-scale complex is recognizably composed of a short axis bearing megasporophylls each with a terminal ovule, and sterile scales. In the Upper Carboniferous and Lower Permian conifers (Florin, 1938–45) the megasporophyll was a discrete organ, but later, in the Upper Permian to Lower Jurassic, in such examples as *Voltzia* and *Schizolepis*, the megasporophyll became somewhat reduced, and was represented by a stalk more or less completely fused to the sterile part of the complex, and an enlarged distal part carrying the seed. This latter part is positionally comparable with the chalazal cushion in *Pseudoaraucaria*, and it seems very reasonable to suppose that the two may be morphologically equivalent. If the cushion does represent part of the megasporophyll, then *Pseudoaraucaria* is more primitive in this respect than any other known member of the Pinaceae.

It is odd that amongst the living Pinaceae only the genus *Pinus* presents a two-ribbed integumental structure similar to that in *Pseudoaraucaria*, for, as has been pointed out elsewhere (Alvin, in press), *Pinus* has perhaps less in common with the fossil group than have some of the other living genera. The significance of this peculiar integumental organization is unknown, but it is possible that it is a primitive feature deriving from the fundamentally platyspermic nature of primitive conifer seeds, where the integument was formed from an opposite pair of scales, each with a single central vascular strand (Florin, 1951).

According to Flous (1936) and Boureau (1939), the number and form of the cotyledons in conifers is of some systematic and phylogenetic significance. Owing to the limitations of the fossil material, it has not been possible to determine which of Boureau's types (A, B, C, or D) the cotyledons in *Pseudoaraucaria loppineti* are. By itself the number of cotyledons (which varies in the family from 2 to 18, and which is often variable within a species) is of no great importance. However, 7 is a very common number, and occurs in all genera of the family except *Cedrus* (9–11), *Keteleeria* (2–4), *Pseudolarix* (4–5), and *Tsuga* (3–4) (Flous, 1936).

It is interesting that certain features of *Pseudoaraucaria* should recall some of the primitive characters of the earlier fossil conifers, for in some respects the group appears specialized. The deeply embedded position of the seeds and the extremely compact form of the whole cone would suggest an evolutionarily advanced condition; moreover, certain structural characters, such as the entirely adaxial distribution of resin ducts and the organization of the tissues in the scale base, also suggest specialization. If *Pseudoaraucaria* represents the cones of a truly natural group of species (for it should be emphasized that at present nothing is known of the trees which bore any of these cones), then it is probably a group which, as it were, split off from the primitive Pinaceous stock at a relatively early stage, and became specialized along certain lines whilst retaining some of the primitive features more characteristic of earlier conifers.

SUMMARY

1. The genus *Pseudoaraucaria* which has been recently revived for a species of Pinaceous cone from the Wealden of Belgium (*Ps. heeri*) is characterized by: (*a*) the cone scale being sharply differentiated into a lower seed-bearing portion and an upper laminoid part set at a sharp angle to the lower; (*b*) the massive interseminal ridge on the scale and the consequent embedded position of the seeds; (*c*) a cushion of parenchyma at the base of the seed. It is suggested that the genus, unlike such wide form genera as *Pinites* Endlicher and *Pityostrobus* Nathorst (both of which in a sense overlap *Pseudoaraucaria*), may represent a relatively small and natural group of species perhaps equivalent to one of the recent genera.

2. The material for which the genus was first erected is now redescribed in some detail. Two species (*Ps. loppineti* and *Ps. major*), instead of three as originally, are recognized. Except in details these species agree structurally with a Belgian species. The seeds, however, are better preserved, and the integument structure and seed contents including the embryo are described in *Ps. loppineti*.

3. Another species, *Ps. benstedi* comb. nov. from the English Lower Greensand, is also redescribed. The cone has the general characters of *Pseudoaraucaria*, but, unlike the other species, the seed integument contains secretory cavities.

4. The possible systematic and phylogenetic significance of the more striking characters are discussed, and it is concluded that the genus combines some features which are probably primitive with others suggesting specialization.

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DEPOSITORIES OF SPECIMENS AND SLIDES

All specimens and slides of *Ps. loppineti* and *Ps. major* are in the collection of the École Nationale Supérieure de Géologie Appliquée, Nancy, France. The type-specimen of *Ps. benstedi* and all slides are in the British Museum (Natural History) collection.

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EXPLANATION OF PLATES II AND III

PLATE II

Pseudoaraucaria loppineti

Figs. 1 and 2. Half of the Type-Specimen. (× 1.)

Fig. 3. A vertical section through the basal part of a scale. Slide D 5. (× 7½.)

Fig. 4. Part of a similar section at a higher magnification, showing the thick sclerotesta and, at the top, one of the two longitudinal sclerotic 'ribs' of the outer layer of the testa. Slide D 6. (× 40.)

Fig. 5. Part of a longitudinal section through the cone showing the base of a scale with one of its seeds cut obliquely. The dark line running from the sclerotesta through the chalazal flesh represents the seed trace. Slide A 10. (× 7½.)

Fig. 6. Part of a strand similar to the one visible in Fig. 5, showing probable scalariform elements. Slide A 7. (× 230.)

Fig. 7. An embryo surrounded by endosperm as seen in a seed cut transversely (tangential to the cone). Photographed under water by reflected light. (× 45.)

PLATE III

FIGS. 1–3. *Pseudoaraucaria benstedi*

Fig. 1. An oblique longitudinal section through the cone. Section in the British Museum (Nat. Hist.) Collection, No. 39107 c. (× 3.)

Fig. 2. Small part of the xylem from the cone-axis in radial longitudinal section, showing uniseriate bordered pits. B.M. Slide No. 39107 h. (× 190.)

Fig. 3. Part of a transverse section through the cone. B.M. Slide No. 39107 n. (× 7½.)

FIGS. 4–6. *Pseudoaraucaria loppineti*

Fig. 4. Part of a transverse section through the cone. Slide C 13. (× 6.)

Fig. 5. Part of a transverse section through the scale showing the tissue abaxial to the vascular strand; at the top, the large, thin-walled cells; in the middle, the dark zone of thick-walled cells; at the bottom, the narrow, thin-walled cells with dark contents. Slide B 7. (× 105.)

Fig. 6. The ovuliferous scale and accompanying bract traces in longitudinal section through the cortex of the axis. Slide E 1. (× 12.)

FIG. 7. *Pseudoaraucaria major*

The ovuliferous scale trace in a section at the extreme base of the scale, showing the sclerotic core. Below can be seen the small bract trace. Slide H 1. (× 20.)

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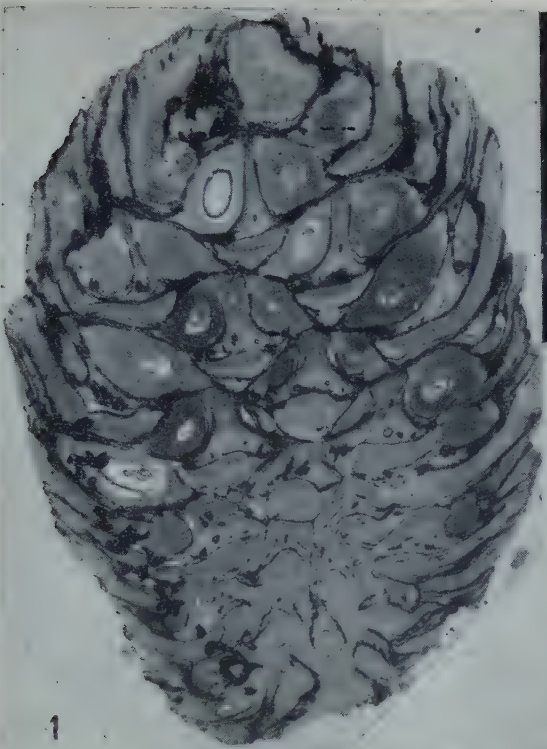
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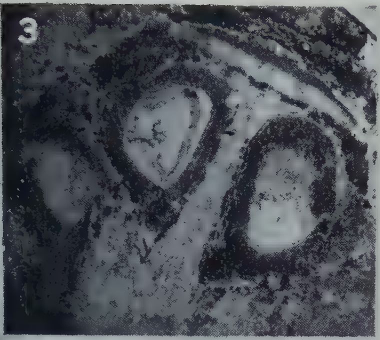
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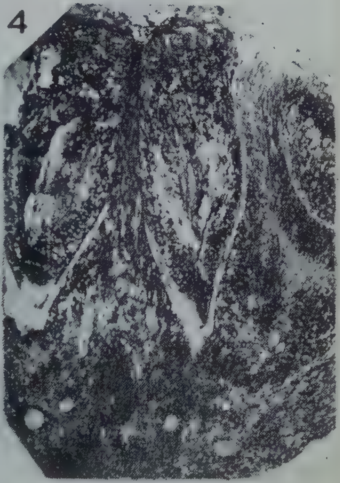
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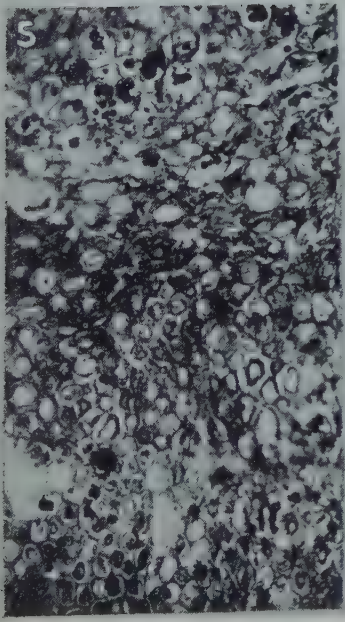
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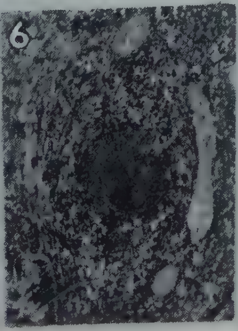
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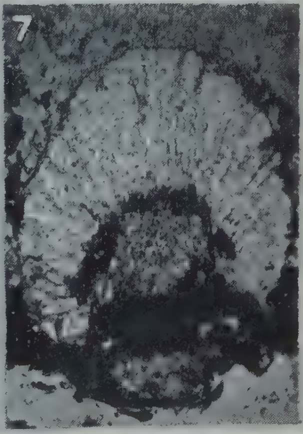
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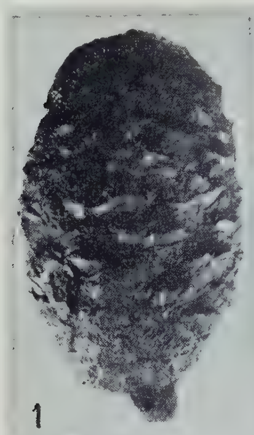
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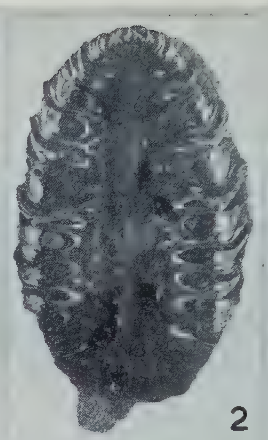
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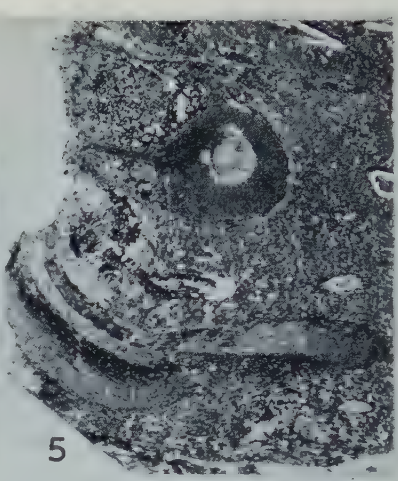
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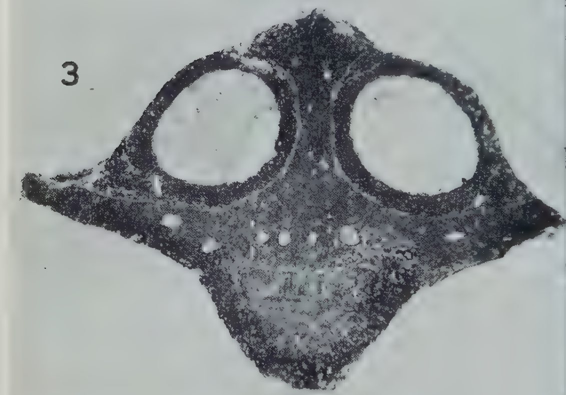
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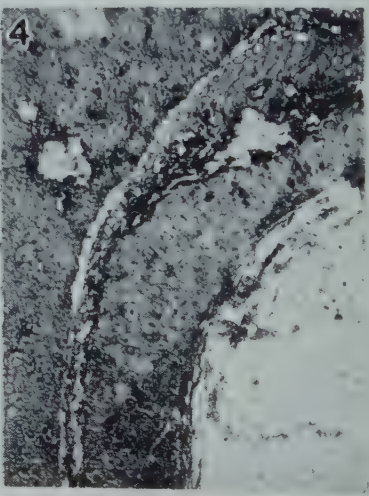
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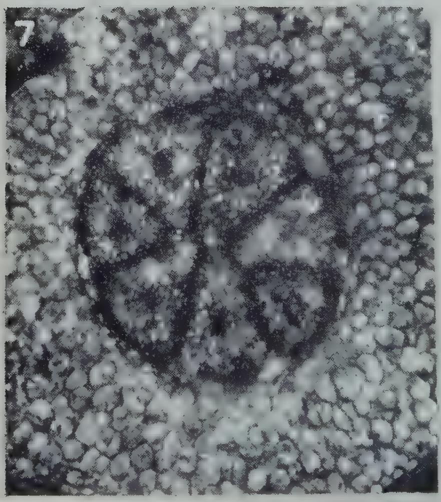
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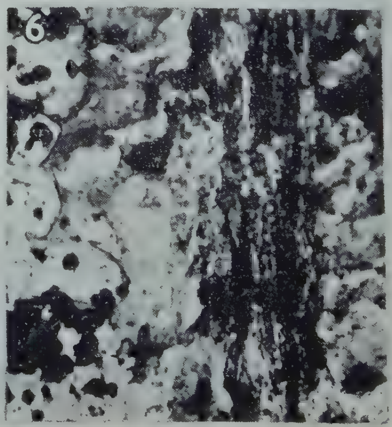
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K. L. ALVIN

Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

I. The Occurrence of Soluble Nitrogenous Compounds

BY

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ABSTRACT

Making use of paper partition chromatography, an investigation has been made of the nitrogenous compounds that occur free in plants which belong to the Liliaceae or, in some cases, to the Agavaceae or to the Amaryllidaceae. Particular attention was paid to the detection of certain substances which have recently been discovered in plants, namely, azetidine-2-carboxylic acid, γ-methyleneglutamine and certain γ-substituted glutamic acids. Maps record the location on chromatograms of the identifiable substances from the plants examined and also of the large number of substances that have been observed but not yet identified. The occurrence of these compounds is considered in relation to the taxonomic classification.

The natural occurrence of the more unusual soluble nitrogen compounds (γ-methyleneglutamine and other γ-substituted glutamic acids, azetidine-2-carboxylic acid) is taken to indicate the existence in plants of hitherto unrecognized metabolic pathways, and their restricted accumulation may result from changes during the course of selection and breeding in some factor or factors governing these pathways.

SINCE the development and use of chromatographic methods the knowledge of the alcohol-soluble nitrogenous compounds that occur in plants has been greatly extended by the identification of a number of substances not hitherto known to occur free and by the recognition of a large number of compounds which are as yet unidentified (Steward, Zacharius, and Pollard, 1955; Virtanen, 1955). Investigators in this field have tended to concentrate upon the isolation of a given compound from a particular plant, or organ, in which its presence had been revealed and later, after the identity has been established, other examples of its natural occurrence were noted. Thus, the new technique was profitably exploited by examining a range of only tenuously related materials—such as a variety of storage organs (roots, tubers, bulbs, and corms), seeds and fruits, leaves of monocotyledons and dicotyledons. In some cases, such as γ-aminobutyric acid, the recognition of the compound in one source (e.g. potato tuber) was rapidly followed by evidence of very widespread, almost universal, occurrence in plants. In others, such as hydroxyproline, the source from which the critical isolation was made may well appear to be a comparatively rare example. The present need now seems to be for more systematic investigations.

The present publications focus attention upon members of a given family of angiosperms and only limited attention is given to some closely related

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plants. The Liliaceae, with its great range of morphological variation, was thought to be a suitable family on which to examine the nature and distribution of the soluble nitrogen compounds present and to ascertain whether this distribution in any way reflects the taxonomic classification. The family was known to include some plants in which unusual nitrogenous compounds appeared. In one genus (*Tulipa*) certain newly discovered and somewhat unusual compounds (γ-methyleneglutamic acid and γ-methyleneglutamine), first identified in *Arachis hypogaea* by Done and Fowden (1952), were known to occur (Zacharius, Pollard, and Steward, 1954) although these were not present in certain closely related plants, e.g. *Allium* and *Asparagus* (Zacharius, 1952). In another genus of the family (*Convallaria*) a new and quite unique type of compound (azetidine-2-carboxylic acid) had appeared (Fowden, 1955). Eighty-nine plant extracts have therefore been made and examined chromatographically from plants belonging either to the Liliaceae, the Agavaceae, or the Amaryllidaceae, all of which have been classified at one time or another with the Liliaceae. In work complementary to the taxonomic survey, one genus (*Tulipa*) was also studied to determine whether the compounds it contained remained fixed and unalterable constituents of the mature plant, or whether they could be modified by changes in the environment and during development.

EXPERIMENTAL

Preparation of the extracts

The more succulent plant materials were extracted at final concentrations of 75 per cent. ethanol after maceration in the Waring Blendor. The seeds were first ground in a Wiley mill and then subjected to the same extraction procedure. The ethanol extraction was continued in the cold until virtually complete removal of ninhydrin reacting materials was obtained. The combined ethanol extracts were then evaporated to dryness at room temperature in a stream of air. In this way the more labile constituents were preserved. The residues were redissolved in a minimum quantity of water and the aqueous solutions were extracted with chloroform to remove pigments. The chloroform-extracted solutions were again evaporated to dryness at room temperature and were subsequently redissolved in such a way that a given volume of the resultant solutions could be related directly to specified weights of the original plant materials. For the leaf material 40 μl. of final solution represented 0·2 g. of fresh leaves, whereas for seeds 40 μl. of the solution represented 0·1 g. of air-dried material. The extracts were stored in stoppered vials with chloroform as a preservative.

Chromatographic methods

The two-directional chromatograms were all made on Whatman No. 3 chromatographic grade papers ($22\frac{1}{4} \times 18\frac{1}{4}$ in.) which were used without preliminary washing. The solvent combinations were phenol saturated with water and adjusted to pH 5·5 in the first direction and butanol-acetic acid in the second. The first solvent was used in the long direction ($22\frac{1}{4}$ in.) of the paper. In order to obtain reproducible chromatograms the conditions were standardized as follows:

Each solvent was allowed to run for 24 hours after the paper had been previously equilibrated in the cabinet to the solvent conditions, all the chromatographic operations being carried out in a constant-temperature room at 75° F. After the chromatography, the papers were treated with ninhydrin in the usual way and the colour of the ninhydrin reactive substances developed in an air oven at 60° C. This procedure is suitable for qualitative observations on the nature of the major constituents present. Notes were also taken of the relative intensities of the spots on the paper: these indicated whether given constituents were major, or minor, constituents of the extracts in question.

Substances that could be readily recognized on the chromatograms as widely occurring constituents of plants were immediately noted by name. Where the papers indicated the presence of other substances, however, these were recorded by number, their positions determined by measurements relative to alanine, and other characteristics of the spots, such as ninhydrin colour, &c., were recorded. Additional to the routine procedure recorded above, certain supplementary techniques were applied where necessary. For example, in order to distinguish between the relatively rare occurrence of hydroxyproline and the occurrence of γ-methyleneglutamine in the same extract, acid hydrolysis was used. Where the general occurrence of pipecolic acid was suspected by its position on the chromatograms, supplementary evidence was obtained from its characteristic fluorescence in ultraviolet light.

The plant materials examined

Extracts were made from plants which fall into 56 genera. These were mainly members of the Liliaceae, but 3 genera are currently classified with the Amaryllidaceae and 6 with the Agavaceae (Bailey, 1949).

In order to present the results of this survey concisely the following conventions have been adopted. First the plants examined are listed in the order in which the genera to which they belong appear in the classification of Engler and Prantl. When a genus was represented by more than one species, each was separately and consecutively listed. The plants as listed were then assigned consecutive numbers (see Table I). In the table 'L' in parenthesis indicates that the leaf was extracted, 'S' in parenthesis that seeds were extracted. If both leaf and seed of one species were extracted the seed bears the next higher consecutive number to the leaf extract. In subsequent references in later tables to the composition of the extracts, the plants will be designated by the numbers assigned to them in Table I.

The data obtained

A number of widely occurring plant constituents appeared on most, if not all, of the chromatograms examined. These substances were not individually noted in the tables but the following amino-acids occurred generally throughout all the extracts: aspartic acid, glutamic acid, serine, asparagine, threonine,

TABLE I

Shows the plant species examined, together with the number assigned to them, and by which they will normally be referred to throughout this paper. The letters in parenthesis indicate which organ was examined, e.g. S = seed; L = leaf.

1 Xerophyllum asphodeloides Nutt (S)
2 Veratrum viride Ait. (L)
3 Gloriosa sp. L. (L)
4 Littonia modesta Hook. (S)
5 Colchicum autumnale L. (L)
6 Asphodeline lutea Reichb. (S)
7 Paradisia liliastrum L. (L)
8 Paradisia liliastrum L. (S)
9 Anthericum liliago L. (L)
10 Arthropodium cirratum R. Br. (S)
11 Bowiea volubilis Hook. (Stem)
12 Hosta lancifolia (Thunb.) Engl. (L)
13 Hosta glauca, Stearn. [H. sieboldiana (Hook.) Engl.] (S)
14 Hemerocallis sp. L. (L)
15 Hemerocallis sp. L. (S)
*16 Phormium tenax Forst. (L)
17 Blandfordia nobilis Sm. (S)
18 Kniphofia sp. Moench (L)
19 Haworthia coarctata Haw. (L)
20 Gasteria verrucosa (Mill.) Haw. (L)
21 Gasteria sp. Duval (S)
22 Aloe barbadensis, Mill. [A. vera L.] (L)
†23 Allium schoenoprasum L. (L)
†24 Brodiaea sp. Smith (S)
†25 Milla biflora Cav. (S)
25*a* Lilium longiflorum Thunb. (L)
26 Notholirion macrophyllum [Lilium macrophyllum Wilson] (L)
27 Notholirion thomsonianum [Lilium thomsonianum Wilson] (L)
28 Fritillaria meleagris L. (L)
29 Fritillaria imperialis L. (S)
30 Tulipa gesneriana L. (L)
31 Tulipa gesneriana L. (S)
32 Tulipa sylvestris L. (L)
33 Tulipa biflora L. (L)
34 Tulipa tarda Stapf (L)
35 Tulipa stellata Hook. (L)
36 Tulipa clusiana DC. (L)
37 Tulipa linifolia Regel (L)
38 Tulipa acuminata Vahl (L)
39 Tulipa fosteriana Hoog (L)
40 Tulipa kaufmanniana Regel (L)
41 Tulipa greigii Regel (L)
42 Tulipa praestans Hoog (L)
43 Tulipa pulchella Fenzl (L)
44 Tulipa biflora var. turkestania [Tulipa turkestania Regel] (L)
45 Tulipa montana Lindley (L)
46 Erythronium sp. L. (L)
47 Erythronium sp. L. (S)
48 Calochortus sp. Pursh. (S)
49 Galtonia candicans Dcne. (S)
50 Dipcadi serotinum (L) Medic. (L)
51 Scilla bifolia L. (L)
52 Camassia sp. Lindl. (L)
53 Camassia sp. Lindl. (S)
54 Eucomis sp. L'Hérit. (L)
55 Ornithogalum nutans L. (L)
56 Drimiopsis maculata Lindl. (L)
57 Chionodoxa luciliae Boiss. (L)
58 Puschkinia sp. Adams (L)
59 Hyacinthus orientalis L. (L)
60 Scilla hohenackeri (Fisch. and Mey.) [Hyacinthus glaucus Baker] (S)
61 Muscari armeniacum Leicht. (L)
62 Muscari armeniacum Leicht. (S)
63 Lachenalia sp. Jacq. (L)
*64 Yucca gloriosa L. (L)
*65 Cordyline terminalis Kunth (L)
*66 Cordyline australis (Forst.) Hook (S)
*67 Dracaena sanderiana Sander (L)
*68 Dracaena deremensis Engl. (L)
*69 Dracaena godseffiana Sander (L)
*70 Dracaena fragrans (L.) Gawl (L)
*71 Sansevieria sp. Thunb. (L)
72 Asparagus sprengeri Regel (L)
73 Danae racemosa (L.) Moench (S)
74 Ruscus aculeatus L. (L)
75 Clintonia sp. Raf. (L)
76 Smilacina racemosa (L.) Desf. (L)
77 Maianthemum canadense Desf. (L)
78 Maianthemum canadense Desf. (S)
79 Polygonatum sp. Adans (L)
80 Convallaria majalis L. (L)
81 Convallaria majalis L. (S)
82 Rohdea japonica Roth & Kunth (L)
83 Aspidistra sp. Ker-Gawl (L)
84 Trillium sp. L. (L)
85 Trillium sp. L. (S)
86 Liriope muscari (Dcne.) Bailey (L)
87 Liriope spicata Lour. (S)
*88 Agave americana L. (L)

Species now generally regarded as belonging to the Agavaceae (*); Amaryllidaceae (†).

It was impracticable to confirm completely the identity of all the materials examined (especially the seeds). In some such cases, therefore, reliance was necessarily placed upon the names assigned by a commercial supplier.

alanine, glutamine, lysine, γ-aminobutyric acid and valine, and the leucines (without distinction between leucine and isoleucine). Other amino-acids occurred less generally. These were: lysine, arginine, proline, phenylalanine, tyrosine, and cysteic acid from the oxidation of cystine. Of the amino-acids that normally arise from protein hydrolysis, methionine and histidine are rather notably absent from the list. The positions on chromatograms of the amino-acids listed above are all shown on the chromatographic map in Fig. 1 and are there represented by open circles.

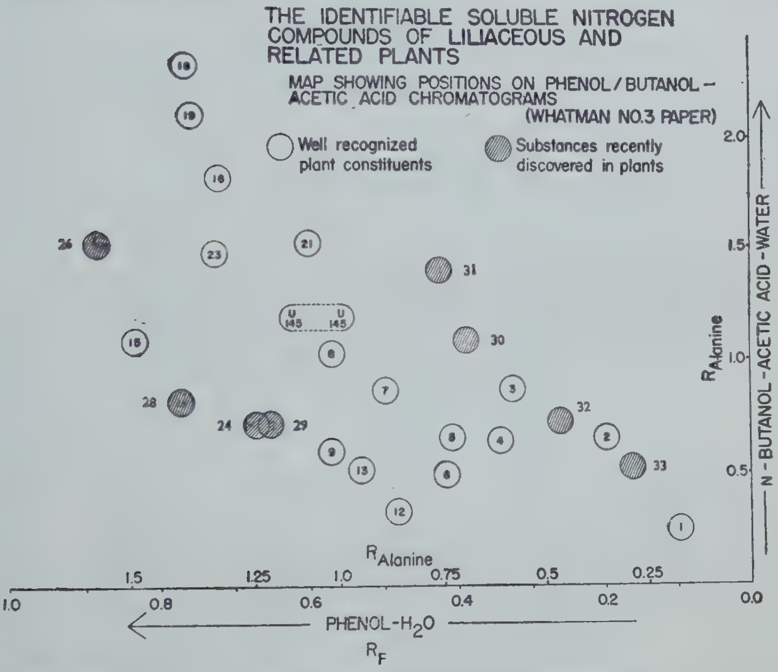

FIG. 1. Map of positions on phenol : butanol-acetic acid chromatograms of identified ninhydrin reactive substances occurring free in Liliaceous and related plants.

○ Amino-acids and amides which occur in proteins or which are well recognized plant constituents.

▨ Recently identified amino-acids.

Key: 1, cysteic acid (from cyst(e)ine); 2, aspartic; 3, glutamic; 4, serine; 5, glycine; 6, asparagine; 7, threonine; 8, alanine; 9, glutamine; 12, lysine; 13, arginine; 15, proline; 16, valine; 18, leucine(s); 19, phenylalanine; 23, γ-aminobutyric; 24, hydroxyproline; 26, pipecolic; 28, azetidine-2-carboxylic; 29, γ-methyleneglutamine; 30, γ-methyleneglutamic; 31, γ-methylglutamic; 32, γ-hydroxy-γ-methylglutamic; 33, γ-hydroxyglutamic; U145, unidentified but generally occurring.

Primary attention will, however, be focused on two other groups of compounds. First, a list of recently identified compounds, and secondly a much longer list of still unidentified compounds which have been detected on the chromatograms. Figs. 1 and 2 record the chromatographic positions of these substances whilst Tables II and III record their occurrence in the plant extracts.

TABLE II

Shows the occurrence of the recently identified amino-acids in the species examined with the R_{Alanine} values and ninhydrin colour reactions of these compounds.

Amino-acid	Nin-hydrin colour	R_{Alanine}		Numbers designating species in which amino-acid occurs. Relative amounts of each amino-acid present:
		Phenol	Butanol-acetic	
γ-methylene-glutamic acid	YB	0·68	1·06	19, 25a 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48 W S T S W T W W W S M T W W W M W W W T W T W Species listed represent 7 genera
γ-methylene-glutamine	YB	1·15	0·70	30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 45, 46, 47 S W M T S S W M S S S S S S M S T Species listed represent 2 genera
γ-methyl-glutamic acid	P	0·75	1·36	25a 26, 27, 30, 31, 32, 33, 35, 36, 37, 38, 39, 40, 41, 42, 43, 47, 48, 58 S W W T W T T T T T T T T T W T T S T Species listed represent 6 genera
γ-hydroxy-γ-methyl-glutamic acid	P	0·46	0·71	4, 25a 30, 32, 33, 34, 35, 36, 37, 38, 39, 42, 44, 46, 48, 58 T T T T T T T T T T T T T T T T Species listed represent 6 genera
γ-hydroxy-glutamic acid	P	0·28	0·51	14, 15, 21 W S S Species listed represent 2 genera
Hydroxy-proline	YB	1·18	0·70	68, 69, 70 T W T Species listed represent 1 genus
Azetidine-2-carboxylic acid	B	1·36	0·79	4, 11, 13, 20, 25, 29, 53, 60, 67, 68, 69, 70, 73, 74, 76, 77, 78, 79, 80, 81, 82, 86, 87 M S W T T T W W S W M M S S S S S S S S S S S Species listed represent 17 genera
Pipecolic acid	Bl	1·56	1·47	12, 19, 29, 57, 59, 61, 76, 77, 81 T T T W M T T W T Species listed represent 9 genera

Ninhydrin colour key: B = brown, YB = yellow-brown; Bl = blue; P = purple.

Amounts of amino-acids present in particular species were qualitatively estimated from the intensities of the ninhydrin-developed spots. The amounts are shown under each number designating species where:

S = strongly reacting spot; M = moderate reacting spot; W = weak reacting spot; T = trace reaction.

TABLE III

Shows the plant species in which the unknown ninhydrin reactive compounds occur. Italic numbers designating a species indicate that this particular species contains the unknown as a major constituent. The table also shows the $R_{Alanine}$ values determined for, and the ninhydrin colour reactions of, the unknown compounds.

Number of unknown compound	Ninhydrin colour	$R_{Alanine}$		Number designating species in which unknowns occur
		Phenol	Butanol-acetic acid	
U101	P	0·97	1·66	25, 29, 81
U102	P	1·10	0·89	2, 24, 29, 65, 77
U103	P	1·11	0·64	8, 10, *14*, *15*, 17, 24, 25, 29, 48, *52*, 60, 65, 73, 81
U104	P	1·30	0·18	2, 10, 13, 14, 15, 21, 23, 25, 29, 46, 59, 60, 66, 75, 77, 79, 85
U105	P	0·24	0·49	14, *15*, *21*
U106	Pi	0·66	0·43	15, 21, 28, 51, 57, 58, 59
U107	P	1·26	0·96	2, 10, 15, 17, 21, 24, 25, 44, 48, 60, 69, 78, 81
U108	P	0·90	1·33	15, 25, 48, 81, 87
U109	P	1·25	1·37	10, 15, *17*, *21*
U110	P	0·89	0·48	15, 25
U111	P	1·24	0·41	14, 15
U112	P	1·30	0·80	14, 15, 75
U113	P	1·23	0·63	2, 4, 10, *16*, 24, 25, 28, 32, 33, 43, 48, 51, 55, 57, 58, 59, 61, 77, 79, 81, 87
U114	P	0·63	1·12	24, 25
U115	Pi	0·48	0·50	2, 17, 28, 51, 57, 59, 60, 61, 87
U116	P	1·28	1·97	8, 60
U117	P	0·46	0·73	4, 30, 32, 33, 34, 35, 36, 37, 38, 39, 42, 43, 44, 46, 48, 58
U118	P	0·47	1·22	48
U119	Y	1·26	1·66	4, 8, 48
U120	Y→YB→P	1·05	0·70	*8*
U121	YB	0·92	1·24	32, 42
U122	P	1·17	1·41	28, 30, 33, 42, 43, 44, 45
U123	RB	1·27	1·58	28, 42, 44, 45, 51, 57, 58
U124	P	0·81	0·60	24, 65, 75, 82, 88
U125	P	1·39	1·34	14, *16*
U126	GBl	1·24	1·29	6, 14, 61
U127	P	1·23	0·92	14
U128	B	1·10	0·52	*23*
U129	P	1·39	0·28	69
U130	P	1·33	0·59	30, 34
U131	YB	1·49	0·90	30, 43
U132	P	0·69	1·98	20, *56*, *64*, 67, 88
U133	P	0·76	1·07	56, 67
U134	P	0·68	0·90	3, 9, *19*, 56, 64, 88
U135	P	1·39	1·14	20
U136	P	1·11	1·98	75
U137	P	1·05	1·43	46, 75
U138	P	1·22	1·07	75
U139	Y	1·22	1·38	24
U140	P	0·75	1·29	27
U141	P	1·13	1·53	9, 85
U142	P	1·42	1·10	6
U143	P	1·13	0·60	*31*
U144	P	0·91	1·50	*31*
U145	P	0·98–1·10	1·15	In nearly all species examined.

P = Purple; Pi = Pink; Y = Yellow; YB = Yellow-brown; B = Brown; RB = Red-brown; GBl = Grey-blue.

The identifiable amino-acids, other than those commonly occurring ones listed above, are shown on Fig. 1 by cross-hatched circles. These substances are: hydroxyproline, pipecolic acid, azetidine-2-carboxylic acid, γ-methyleneglutamine, γ-methyleneglutamic acid, γ-methylglutamic acid, γ-hydroxy-γ-methylglutamic acid. Although as yet unidentified, the substance designated U145 is included on Fig. 1 because it appeared almost universally throughout these extracts. The positions on chromatograms of these more unusual but identifiable compounds are given more quantitatively in Table II by their R_F values, with reference to alanine, together with observations on their reaction with ninhydrin.

TABLE III*a*

Unidentified Substances Recognized in Lilium

No. of unknown compound	Ninhydrin colour	$R_{Alanine}$ Phenol	$R_{Alanine}$ Butanol acetic acid
U145	P	1·00	1·04
U146	B	0·39	0·57
U147	B	0·20	0·24
U148	RB	0·31	0·74
U149	P	0·55	0·40
U150	P	0·21	0·63
U151	P	0·20	0·34
U152	P	0·08	0·28
U153	P	0·08	0·18

(A spot also occurs in the general vicinity of U109, U139, and U126)

The large number of still unidentified substances that have been detected on the chromatograms are listed by numbers ranging from U101 to U145. The positions of these substances on chromatograms are shown on Fig. 2. The positions as recorded on the map represent the averages of all the information obtained during the survey and the mean R_F values with reference to alanine, together with observations on the behaviour of the substances with ninhydrin, are recorded in Table III. Tables II and III also indicate the distribution of these substances throughout all the extracts of the plants examined.

Qualitative observations on the relative concentrations of the identifiable substances in plants in which they occur are also noted in Table II, together with an indication of the number of genera in which a particular substance has been observed.

In Table III, recording the occurrences of the still unidentified substances, a further convention was adopted. Where the substance occurred as a major constituent of a species, the number of that species is shown in italic.

When the data of the survey were assembled it became clear that two species (Nos. 26 and 27 of Table I) do not now belong, as first thought, to the genus *Lilium* but are currently classified (see Woodcock and Stearn, 1950) in a separate genus (*Notholirion*). Therefore, in order to have the genus *Lilium* represented in the survey, extracts were made, and similarly treated, using leaves from lilies in commercial production: these were classified as *L. floridii*, *L. croft*, and *L. erabu*, all of which may be regarded as clones of *L. longiflorum* which is now designated at 25*a* in Table I. In all major respects the extracts from these three *Lilium* materials were similar. A diagram showing the location of the spots obtained on typical chromatograms from *Lilium* is given at Fig. 3 and the details of additional unknown substances that require to be designated by numbers (U146 to U153) are appended to Table III as Table III (*a*).

Discussion of the Results

The range of ninhydrin reactive substances encountered in this survey can readily be seen from Figs. 1, 2, and 3. It should be emphasized that these comprise about sixty substances other than those which are normally recognized as products of protein hydrolysis. Of these, however, eight are now identifiable as substances not hitherto regarded as widely occurring in plants. Tables II and III indicate the extent to which these substances occur generally throughout the plants examined.

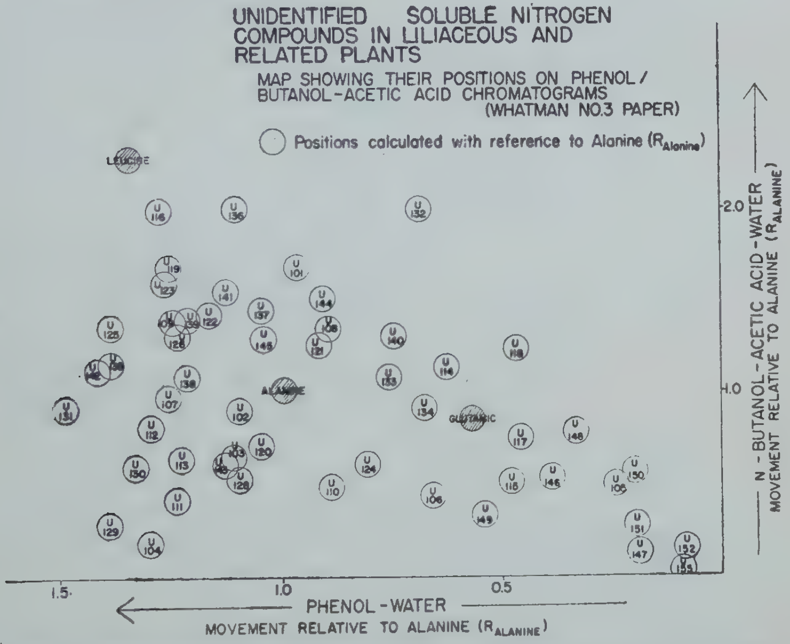

Fig. 2. Map of positions on phenol : butanol-acetic acid chromatograms of unidentified ninhydrin reactive substances occurring free in Liliaceous and related plants.
(For key to the occurrence of U101–U145, $R_{Alanine}$, ninhynrin colour see Table III.)

Before discussing the occurrence of the more unusual but identifiable substances encountered in this survey the following comments on their chemical relationships can be made. Six of the compounds bear obvious relationships to glutamic acid. Four of these, γ-methyleneglutamine and γ-methyleneglutamic acid, γ-methylglutamic acid and γ-hydroxy-γ-methylglutamic acid, are all 6-carbon acids with a carbon substituent on the γ-carbon. γ-methyl-γ-hydroxyglutamic acid can be related to the γ-methylene compounds by the addition of a molecule of water and the γ-methylglutamic acid by reduction. Two other substances, γ-hydroxyglutamic acid and hydroxyproline, both 5-carbon acids like glutamic acid, bear the same general relationship to each other as glutamic acid bears to proline.

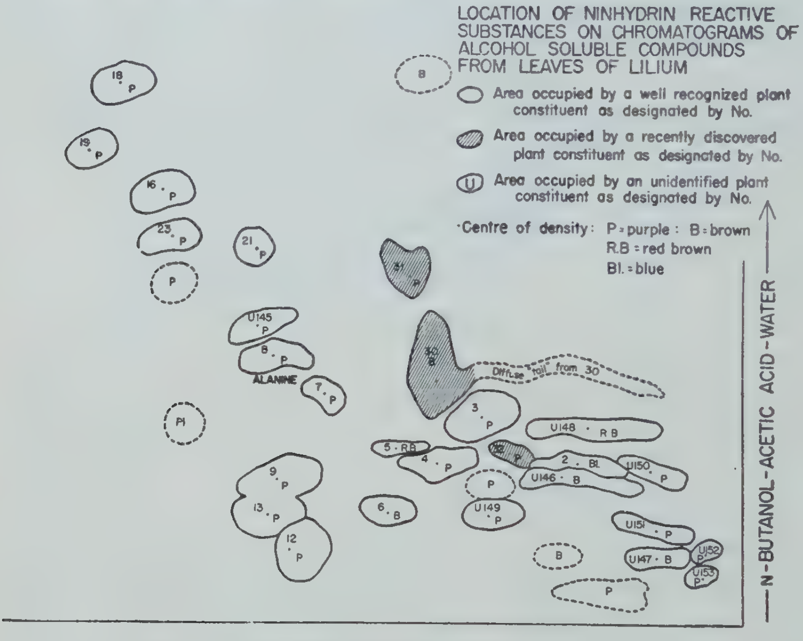
LOCATION OF NINHYDRIN REACTIVE SUBSTANCES ON CHROMATOGRAMS OF ALCOHOL SOLUBLE COMPOUNDS FROM LEAVES OF LILIUM
Area occupied by a well recognized plant constituent as designated by No.
Area occupied by a recently discovered plant constituent as designated by No.
U Area occupied by an unidentified plant constituent as designated by No.
·Centre of density: P = purple : B = brown
R.B = red brown
Bl. = blue
18 P
19 P
16 P
23 P
21 P
P
B
31 P
U145 P
8 P
ALANINE
7 P
30 B
Diffuse tail from 30
P1
3 P
U148 RB
5 RB
4 P
32 P
2 BL
U150 P
U146 B
9 P
13 P
12 P
6 B
P
U149 P
U151 P
U152 P
U147 B
U153 P
B
P
N-BUTANOL-ACETIC ACID-WATER
PHENOL-H2O

FIG. 3. Location of ninhydrin reacting compounds on phenol: butanol-acetic acid chromatograms of the alcohol soluble fraction of the leaves of *Lilium*.
(For key to identifiable substances see Fig. 1.)
(For key to unidentified substances see Table III*a*.)

It is possible that the recently discovered azetidine-2-carboxylic acid may also be related to the γ-methyleneglutamic compounds, for a conceivable oxidation product of γ-methyleneglutamic acid could be aspartic-β-semialdehyde. This in turn could be regarded as a possible precursor of azetidine-2-carboxylic acid by a process of cyclization.

Although pipecolic acid (piperidine-2-carboxylic acid) has been included with the identifiable but more unusual constituents of these extracts it bears no direct chemical relationship to the other compounds listed in Table II. It is, however, interesting that in the sequence pipecolic acid, proline, azetidine-2-carboxylic acid, we have a series of analogous compounds all of which may eventually be found to have similar dicarboxylic and diamino precursors, in the same way that lysine gives rise to pipecolic acid (Grobbelaar and Steward, 1953) and ornithine and glutamic acid are known to give rise to proline.

The nature of the evidence on which these substances were identified on the chromatograms may be summarized as follows. In all cases reliance had to be placed upon the chromatographic position, the reaction with ninhydrin and, where possible, co-chromatography with a substance of known constitution. The chemical identities of γ-methyleneglutamine and γ-methyleneglutamic acid (Done and Fowden, 1952; Wailes, Whiting, and Fowden, 1954), azetidine-2-carboxylic acid (Fowden, 1955) and pipecolic acid have been definitely established (Zacharius, Thompson, and Steward, 1954). The recent identification of γ-hydroxy-γ-methylglutamic acid, found to occur in certain ferns, is recorded by Grobbelaar, Pollard, and Steward (1955). The evidence for the γ-hydroxyglutamic acid rests upon a recent observation that it occurs as a plant constituent (Virtanen and Hietala, 1955) plus the fact that a synthetic product, obtained from Dr. L. P. Bouthillier, University of Montreal, co-chromatographs with the substance first designated U105 in the tables, which is now to be regarded as identified substance No. 33 as shown on Fig. 1. Further, U 103 is converted to U105 by hydrolysis with N—HCl for 3 hours at 100° C., conditions normally used to hydrolyse an amino-acid amide. Whilst in a survey of this kind it is clearly impossible to submit every substance designated to exhaustive isolation and analysis, it is still regarded as desirable that the bulk isolation of the γ-methyl-γ-hydroxyglutamic acid and the γ-hydroxyglutamic acid should eventually be attempted from these Liliaceous plants.

Before attempting a more detailed analysis of the occurrence of the nitrogenous constituents throughout the plants examined, the following rather general comments can be made.

(i) Alanine was universally distributed and tended to be the major free amino-acid in these plants.
(ii) Glutamic acid and serine also occurred universally and in relatively high concentrations, and aspartic acid, though generally present, occurred in smaller amount.
(iii) The occurrence of γ-aminobutyric acid was somewhat erratic, ranging from virtual absence to its occurrence as a major constituent.
(iv) Of the two amides glutamine predominates. It was present in all extracts whereas asparagine was not detected in one-quarter of the total number.

Seeking amongst the less familiar nitrogenous constituents for a substance that might be typical of the whole group of these Liliaceous plants, it would seem to be most readily found in the still unidentified substance designated U145 which appears to be of almost universal occurrence throughout the group. Other less prevalent unknowns do show a tendency to occur in closely related plants, e.g. U106, U113, U115, and U123 all occur in *Scilla* (51) and *Chionodoxa* (57). Whereas one might have supposed that the γ-methylene compounds, which were early recognized to occur in the tulip, might have emerged as somewhat typical of the family, this is far from being the case since these substances are restricted to *Tulipa* and closely related genera. On the other hand the newly discovered compound, azetidine-2-carboxylic acid, emerges as a rather widely occurring substance throughout this family of plants. The more specific comments to be made are as follows:

Azetidine-2-carboxylic acid may occur in all parts of a plant (leaf, stem, root, seed), as in fact it does in *Convallaria*. This is not invariably so as it may occur in the seed but not in the leaf (*Camassia*, *Fritillaria*, and *Hosta*), whilst in other cases it may occur in the leaf but not appear in the seed, as for example in *Gasteria*. There is some indication that azetidine-2-carboxylic acid tends to occur in the leaves of certain closely related plants, this being shown by the fact that it has been recognized in extracts made from many of the species numbered between 67 and 87 (see Table II) and, from the method of presentation, it will be evident that these tend to be plants rather closely related within the family. However, outside the range of species so indicated there are other occurrences of azetidine-2-carboxylic acid which appear to extend throughout the whole range of plants examined, but these examples are mainly of occurrence in seeds. Examples of this are as follows: *Littonia*, *Hosta*, *Milla*, *Fritillaria*, *Camassia*, and *Scilla*, though the substance also occurs in the stem of *Bowiea* and in the leaf of *Gasteria*.

Referring now to the occurrence of γ-methyleneglutamine and γ-methyleneglutamic acid it is apparent that, where both substances occur, the amide tends to predominate over the free acid. On the other hand the γ-methyleneglutamic acid seems to occur in more genera than does the amide (the free acid being reported from seven genera whereas the amide is as yet only reported from two). These genera are all closely related to *Tulipa* and are: *Tulipa*, *Lilium*, *Notholirion*, *Fritillaria*, *Erythronium*, *Calochortus*, and *Haworthia*, where the latter is more distantly related.

If the γ-methylglutamic acid and the γ-hydroxy-γ-methylglutamic acid are to be regarded as being derived from the γ-methylene compounds, one would expect their distribution to be similar to that of the γ-methylene compounds or to be even more restricted. This is apparent in the case of the γ-methylglutamic acid for its distribution seems to be the same as that of γ-methyleneglutamic acid. In the case of γ-hydroxy-γ-methylglutamic acid the distribution is also similar to that of the γ-methyleneglutamic acid with two notable exceptions. γ-hydroxy-γ-methylglutamic acid is present, unaccompanied by γ-methyleneglutamic acid, in *Littonia* and *Puschkinia*. The two latter examples are similar to the case of *Adiantum* in which γ-hydroxy-γ-methylglutamic acid is present in large amount although, so far as at present known, γ-methyleneglutamic acid does not occur in that plant.

Fig. 3 shows that *Lilium* contains γ-methyleneglutamic acid (30), γ-methylglutamic acid (31), and γ-hydroxy-γ-methylglutamic acid (32). Of these the substance present in outstanding amount was γ-methyleneglutamic acid. The presence of γ-hydroxy-γ-methylglutamic acid was carefully confirmed by co-chromatography. A further substance in *Lilium* occupied a position on the chromatograms near to that of γ-hydroxyglutamic acid, but co-chromatography with authentic γ-hydroxyglutamic, during which the phenol solvent was allowed to run considerably longer than usual, effected a separation of the substance (U151, see Fig. 3) in *Lilium* and γ-hydroxyglutamic acid. Thus U151 was not γ-hydroxyglutamic acid, though it may be some other hydroxy acid. It is thus evident that *Notholirion* resembles *Lilium*, from which it is now separately classified, in that γ-methyleneglutamine does not accumulate in either, but it differs from *Lilium* in the very weak representation of all the γ-substituted glutamic acids, since γ-methylene- and γ-methylglutamic acids occur (conspicuously) in *Lilium* but not appreciably in *Notholirion*. There are also a number of unidentified substances (U146–U153 in Table III*a*) which occur in *Lilium* but not in *Notholirion*, whilst still other substances appeared as faint spots too ill defined to warrant their separate designation, though they are represented by dotted lines on the diagram at Fig. 3.

The genus *Fritillaria* presents a somewhat unusual situation in that γ-methyleneglutamic acid occurs in the leaves of one species, *Fritillaria meleagris*, unaccompanied by azetidine-2-carboxylic acid, whereas in another species, *Fritillaria imperialis*, the seed showed the presence of the azetidine-2-carboxylic acid unaccompanied by the γ-methyleneglutamic acid.

A final conclusion would seem to be as follows: ability to synthesize or even to accumulate the more unusual or distinctive soluble nitrogen compounds here considered seems to occur rather widely throughout the range of plants examined. This, together with the sporadic examples that have been noted of their occurrence in plants far removed from the Liliaceae (γ-methyleneglutamine, &c., in *Arachis*; γ-hydroxy-γ-methylglutamic acid in ferns; γ-hydroxyglutamic acid in *Phlox*) suggests that the factors governing their distribution do not operate at the generic or even the specific level. (In short, the accumulation of any one of these compounds may be determined by relatively few of the genes that characterize the organism.)

The occurrence of these compounds, therefore, should now be regarded as evidence of the widespread existence of metabolic pathways which had not previously been suspected. In most plants the occurrence of these substances will be, or may be, far below the limits which permit their detection; thus the amount of carbon they represent, or which normally flows through them, may be slight. In other plants, however, by virtue of factors that probably operate at the genetic level and therefore may be greatly affected by selection in cultivated plants, or to special conditions that are associated with given states of development and thus may be affected by environment and nutrition, these substances accumulate to the point where detection is not only possible but their presence becomes obtrusive. A full understanding of the nitrogen metabolism of plants therefore requires that the biogenesis of these compounds and the factors that govern their accumulation should be investigated.

SUMMARY

1. A survey has been made of the alcohol-soluble nitrogen compounds which can be separated by partition chromatography on paper and detected by ninhydrin and that occur in the Liliaceae and certain related plants.

2. Maps locate the positions on chromatograms of the substances that occur free in these plants. One such map records the more familiar compounds (such as the amino-acids of protein hydrolysis and their amides of which eleven occur generally in the family and another seven were frequently noticed) plus a number (8) of identifiable but still unusual compounds, namely: hydroxyproline, pipecolic acid, azetidine-2-carboxylic acid, γ-methyleneglutamine, γ-methyleneglutamic acid, γ-methylglutamic acid, γ-hydroxy-γ-methylglutamic acid, and γ-hydroxyglutamic acid. Further maps plot the positions of a large number (53) of substances that still remain to be identified.

3. The distinctive features of the plants examined were sought in the occurrence in these eight recently identified nitrogen compounds and in the still unidentified compounds that were detected.

4. Whereas γ-methyleneglutamine by virtue of its early discovery in *Tulipa* might have been expected to be typical of the family, this is not so, for this substance, and its close relatives, now appear to occur mainly in *Tulipa* and in certain of the more closely related genera.

5. Azetidine-2-carboxylic acid, which can occur in leaf, stem, and root, as in *Convallaria*, seems to occur in the leaves of a large number of somewhat closely related members of Liliaceae but its distribution is even more widespread in seeds.

6. Although the amide γ-methyleneglutamine when present is commonly in excess of its free acid, the latter is the more widespread substance. As expected, other substances that bear a chemical relationship to the γ-methylene compounds also show a similar pattern of distribution indicating some similarity in their biogenesis.

7. One of the unidentified substances (U145) is of special interest because it seems to occur almost universally in these plants although it appears not to have been widely reported from other plant families.

8. Three leaf samples from the genus *Lilium* were strikingly similar to each other but different from *Notholirion* in certain respects. Certain unidentified compounds (U146–U153), not observed in the other plants sampled, were, however, encountered in *Lilium*. The γ-substituted glutamic acids (particularly γ-methyleneglutamic acid) were conspicuous in *Lilium*, but γ-methyleneglutamine was not detected.

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Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

II. The Nitrogenous Compounds of Leaves of the Genus *Tulipa*: Environmental Effects on the Composition of *Tulipa gesneriana*

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ABSTRACT

Because of the predominance of γ-methyleneglutamine in *Tulipa gesneriana* a quantitative, chromatographic investigation has been made to show the factors which determine the composition of the alcohol-soluble nitrogen fraction in *Tulipa*.

A comparative study of the amino-acids that occur free in the leaves of fourteen different species of *Tulipa* has been made. γ-methyleneglutamine varied from as much as 60–70 per cent. of the amino-acid nitrogen down to levels below those which permit its chromatographic detection.

The effects of temperature during growth and of diurnal variation on the nitrogenous compounds of the leaves of *T. gesneriana* are described.

Excised shoots of *T. gesneriana* were exposed to light and darkness to affect the balance of protein synthesis and breakdown so that the concomitant effects on the γ-methyleneglutamic acid compounds and other main components of the soluble nitrogen fraction could be determined.

Lastly, the relative ease of entry of C^{14} from $C^{14}O_2$ into the sugars and certain other compounds of *T. gesneriana* is contrasted with the relative difficulty with which it enters the γ-methyleneglutamine of the *mature* leaves on *excised* shoots.

The occurrence and metabolism of γ-methyleneglumatine in *T. gesneriana* leaves may, therefore, be regarded as additional to and superimposed upon an otherwise typical pattern of nitrogen metabolism: in these leaves γ-methyleneglutamine does not assume the role usually discharged by glutamine.

THIS more detailed study of the nitrogenous compounds present in the leaves of the genus *Tulipa* was made for the following reasons:

Amongst the Liliaceous plants which have been examined, *Tulipa gesneriana* was somewhat unusual in the amounts of the γ-methyleneglutamic acid compounds which it contains. In fact *Tulipa* is the only example other than that of *Arachis hypogaea* in which these compounds are known to occur in sufficiently large amounts to suggest that metabolic studies would be profitable. Fowden (1954) had already examined certain of the metabolic relationships of these newly discovered compounds in *Arachis* with a view to determining their possible functions. It seemed, therefore, suitable to attack similar problems by utilizing the particular features presented in *Tulipa gesneriana*, hence an attempt has been made to determine the factors which affect γ-methyleneglutamine, γ-methyleneglutamic acid, and the other amides and amino-acids with which they should be considered.

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Prior to the more detailed examination of *Tulipa gesneriana*, however, it was desirable to ascertain the variation which occurs within the genus using such other *Tulipa* species as were available. The taxonomic relationships within the genus *Tulipa* are obviously complex. According to Hall (1940) the genus may be divided into a large number of sections and subsections and includes subspecies numbering at least seventy. Of these different subspecies fourteen have been examined: these represent diploids with a chromosome number of 24, tetraploids (48), and a possible pentaploid (60). While this is only a partial examination of the family it does permit the range of composition encountered in the nitrogen compounds to be discerned.

Since other important investigations in the field of nitrogen metabolism have utilized monocotyledonous plants, added interest was attached to this investigation. Amongst these earlier studies the well-known investigation of *Narcissus* may be cited (Vickery, Pucher, Wakeman, and Leavenworth, 1946), and numerous investigations on the metabolism of starving leaves, particularly excised leaves of barley (Yemm, 1937, 1949, and 1950). Of necessity, however, the earlier investigations, lacking the resolving power of the chromatographic techniques, made reference only to broad categories of nitrogen compounds. It was, therefore, of particular interest to re-investigate the factors that regulate the nitrogen compounds in a monocotyledonous leaf, especially one containing the unusual substances described, and to study in detail the range of metabolites that could be determined by the chromatographic methods. Furthermore, experiments have been made utilizing $C^{14}O_2$ to obtain some preliminary information concerning the accessibility of the γ-methyleneglutamic acid compounds to labelling with C^{14}. Although the ultimate objective, namely, the obtaining of these compounds labelled with C^{14} at high specific activities, was certainly not achieved, nevertheless some useful information regarding the general metabolism of these compounds was obtained and will be reported.

EXPERIMENTAL

Methods

The extraction procedure adopted up to the stage of the concentrated aqueous extract used for paper chromatography was the same as that described in the preceding paper. However, in this work the chromatographic methods were modified to permit quantitative results. The soluble nitrogen compounds were quantitatively estimated by the general procedure which has already been described (Thompson, Zacharius, and Steward, 1951; Thompson and Steward, 1951). In the interim, however, the following modifications have been made and apply to this particular investigation.

The chromatograms were made using Whatman No. 3 chromatographic grade paper and the solvent combinations were phenol-water : butanol-acetic acid. These conditions are different from those previously adopted in the method as first described but calibration curves of all the substances estimated were either available or they were specially determined for the purposes of this investigation. While most of the amino-acids were determined by measuring their ninhydrin colour at a wave-length of 570 mμ, asparagine was normally determined at a wave-length of 330 mμ. It was found necessary to determine γ-methyleneglutamic acid by its ninhydrin colour at a wave-length of 340 mμ and γ-methyleneglutamine at a wave-length of 410 mμ. A still unidentified substance (U131 on Fig. 2 of Part I) was also determined at a wave-length of 410 mμ in those cases where it appeared. For purposes of assigning an arithmetical value to the content of the substance U131, it was assumed that it had a molecular weight of the order of 100 and that its reaction with ninhydrin was similar to that of γ-methyleneglutamine. Free ammonia in the extracts was determined by distillation and titration following Conway (1939). Total soluble nitrogen was determined by usual Kjeldahl digestion procedures and the final estimation of ammonia was done by the Conway procedure. The combined chromatographic and analytical procedures rendered a fairly complete account of the total soluble nitrogen in the extracts. The nitrogen recovered by ninhydrin plus the free ammonia normally represented about 80 to 85 per cent. of the total Kjeldahl soluble nitrogen.

The range of composition of leaves in the genus Tulipa *with respect to nitrogen compounds*

Leaf samples from mature plants bearing flowers were obtained from plants belonging to fourteen of the *Tulipa* subspecies. These plants as examined represent seven out of the twelve groups of subsidiary species into which Hall classifies the genus *Tulipa*. The nitrogenous composition of the leaves of these plants is recorded in Table I, each amino-acid being represented by the percentage which its α-amino-nitrogen contributes to the total α-amino-nitrogen determined by the ninhydrin procedure. In some cases the amount of leaf material available for analysis was limited, so that the chromatograms were made on an amount of extract too small to reveal all the minor constituents. Probably if these determinations had been repeated on larger amounts some of the amino-acids not recorded in Table I would have been found to be present.

The outstanding feature of the results recorded in Table I relates to the occurrence of γ-methyleneglutamine. This substance ranges from virtual absence, as in *Tulipa turkestanica*, to an outstanding quantity as in *Tulipa acuminata* and *Tulipa gesneriana*, approximately 70 per cent. of the total α-amino-nitrogen of the leaf. Other species represent γ-methyleneglutamine contents throughout the whole of this percentage range. On these data alone there seems to be no obvious correlation with the chromosome complements of these plants and the only correlation with the taxonomic classification in the genus is that those species which contain the very large amount of γ-methyleneglutamine belong to two subsections (the Gesnerianae and the Eichleres of Hall), both of which are classified in the same section of the family (the Leiostemones). At this point, therefore, one might regard the great

TABLE I

Shows the relative amounts of amino-acids occurring in the leaves of different species of *Tulipa*. The nitrogen content of each amino-acid is expressed as a percentage of the total nitrogen present in all amino-acids determined for any species as obtained by summation.

	T. sylvestris	*T. tarda*	*T. stellata*	*T. clusiana*	*T. gesneriana*	*T. acuminata*	*T. fosteriana*	*T. kaufmanniana*	*T. greigii*	*T. praestans*	*T. pulchella*	*T. turkestanica* [*T. biflora* var. *turkestanica*]	*T. linifolia*	*T. montana*
Aspartic acid	9·6	2·5	2·9	12·3	1·5	1·0	1·4	0·9	3·2	0·5	2·1	3·9	2·5	5·8
Glutamic acid	15·6	9·9	10·4	20·9	2·5	3·2	2·3	2·2	4·2	1·5	4·7	9·7	10·9	18·0
Serine/glycine	T	8·8	5·9	10·2	2·6	2·3	11·6	4·5	2·7	9·8	7·5	9·3	4·4	3·2
Asparagine	T	4·6	10·4	8·2	5·6	3·4	5·9	7·8	6·1	5·6	4·9	19·9	15·6	29·8
γ-methyleneglutamic acid .	T	1·5	7·3	7·1	1·0	4·1	1·2	1·8	6·9	1·5	T	T	T	9·7
Threonine	—	2·3	T	—	0·4	0·8	2·5	—	—	1·9	2·4	2·8	2·3	1·8
Alanine	21·4	22·3	17·0	9·8	1·3	11·4	16·5	10·7	2·2	16·5	17·3	24·9	18·5	2·4
Glutamine	20·1	15·6	7·2	26·0	12·4	3·9	17·5	15·7	16·7	16·0	23·5	17·1	21·9	10·5
γ-methyleneglutamine . .	27·8	24·8	36·5	5·5	71·9	69·6	24·5	51·9	57·6	40·6	26·6	—	17·1	9·9
Valine	5·6	2·1	—	—	0·3	—	5·6	0·9	0·3	2·1	3·7	5·3	2·2	1·8
Leucines	T	2·4	—	—	0·4	—	10·7	—	—	2·0	7·0	7·0	3·8	7·1
γ-aminobutyric acid . .	—	3·2	2·3	—	—	—	—	3·2	—	1·2	T	—	—	—
γ-methylglutamic acid . .	—	—	—	—	—	0·3	0·2	0·3	—	0·6	—	—	—	—
γ-hydroxy-γ-methylglutamic acid	—	—	—	—	—	—	—	—	—	—	0·2	—	0·9	—

T = Substances present but in amounts too small to assay.

predominance of γ-methyleneglutamine in the leaves of *Tulipa gesneriana* as a biochemical genetic characteristic which has resulted from breeding and selection in this species.

The content of γ-methyleneglutamic acid also varies throughout the group of species examined, from quantities too small for detection up to about 10 per cent. of the total α-amino-nitrogen determined. Although the γ-methyleneglutamic acid contents were normally low, some species, with low γ-methyleneglutamine contents, contained the acid in amounts equal to, or greater than, that of the amide, e.g. *Tulipa clusiana*. Although γ-methylglutamic acid was detected in four species and γ-hydroxy-γ-methylglutamic acid in two, neither of these substances ever occurred in amounts greater than one per cent. of the total amino-nitrogen present.

It is clear that no reciprocal relationship exists between γ-methyleneglutamine and the other amides asparagine and glutamine. In short, when the content of γ-methyleneglutamine is quite large, for example, of the order of 70 per cent. as in *Tulipa gesneriana*, the total α-amino-nitrogen present tends to be correspondingly high. Comparing the contents of glutamine and asparagine in the leaves of *Tulipa* it is found, in the usual case, that glutamine is in excess of asparagine; this is not a universal feature because in *Tulipa montana* the asparagine content was approximately three times the glutamine content. It should be noted here that this condition is the reverse of that which has been observed in *Arachis*, the only other plant containing γ-methyleneglutamine in quantity which has yet been investigated in detail. In *Arachis*, where γ-methyleneglutamine is the major amide, asparagine predominates markedly over the glutamine; thus it appeared that γ-methyleneglutamine assumed some of the roles that glutamine normally plays in plants. Of the other amino-acids that contribute to the soluble nitrogen, aspartic acid, glutamic acid, and alanine tend to predominate but here again the range of values that may be encountered is wide. The content of γ-aminobutyric acid in the leaves of tulip tends to be strikingly low and, in the range of plants examined, it was only detected in five species and never represented more than 3 per cent. of the total amino-nitrogen.

Whilst it might be suggested that the wide variation in the content of γ-methyleneglutamine in the range of plants examined was the result of environmental factors, rather than specific or genetic differences, this is unlikely because, in the detailed experiments to follow utilizing *Tulipa gesneriana*, it was peculiarly difficult to affect greatly the content of γ-methyleneglutamine even though the leaves were subjected to a wide range of environmental conditions.

Effects of diurnal variation and temperature during growth on the leaf composition of Tulipa gesneriana

For these experiments *Tulipa gesneriana* plants were used. The plants had been temperature treated so that the growth of the floral shoot had actually commenced prior to the experimental procedure, the shoots having emerged

TABLE II

Shows the relative amounts of various nitrogenous compounds present in *Tulipa* leaves grown under differing conditions of temperature and diurnal variations. Data are expressed as micrograms α-amino-N per gram fresh weight of leaf for the amino-acids, and as micrograms N per gram fresh weight present in ammonia and total soluble nitrogen. Each figure documented is the mean of determinations on two separate samples.

	Dark 40° F. 13 days	Dark 50° F. 13 days	Dark 60° F. 13 days	Dark 70° F. 13 days	Light 40° F. 13 days	Light 50° F. 13 days	Light 60° F. 13 days	Light 70° F. 13 days	Dark 40° F. 52 days	Dark 50° F. 37 days	Dark 60° F. 20 days	Light 40° F. 52 days	Light 50° F. 37 days	Light 60° F. 20 days
Aspartic acid	11·9	12·0	7·6	8·0	17·0	14·8	6·4	8·9	11·8	15·1	7·2	16·1	15·5	9·9
Glutamic acid	24·2	16·5	7·7	7·0	34·8	30·4	20·7	19·5	24·9	17·5	9·0	28·8	20·5	17·5
Serine/glycine	5·3	10·8	10·8	26·3	4·7	5·7	4·9	19·7	5·8	6·5	14·1	6·7	10·8	13·6
Asparagine	0·0	3·6	9·1	18·0	0·0	6·6	6·6	15·8	0·0	6·1	8·5	0·0	5·4	6·6
γ-methyleneglutamic acid .	4·1	3·0	6·5	8·3	5·1	6·9	6·0	9·0	4·1	4·7	3·1	4·0	3·7	2·6
Threonine	2·4	2·1	1·9	2·4	1·2	1·0	1·0	2·8	2·1	2·1	2·1	1·2	1·4	1·7
Alanine	6·4	2·7	3·9	29·7	7·0	4·9	4·8	28·5	6·3	6·4	8·6	5·9	6·3	8·0
Glutamine	34·0	61·6	81·4	87·2	47·2	79·2	40·8	62·6	34·3	65·5	50·5	43·0	38·1	53·9
γ-methyleneglutamine . .	185	185	189	217	253	238	194	213	178	177	183	217	172	211
Lysine	1·3	0·7	1·8	2·4	1·1	1·6	1·3	2·2	1·1	2·4	1·5	1·0	2·0	1·5
Unknown U131 . . .	7·7	2·9	1·7	Trace	8·5	3·2	1·6	0·0	7·2	3·0	1·7	8·3	3·4	1·8
Ammonia-N	17·6	22·2	37·7	44·7	17·6	22·0	15·5	23·5	18·9	27·3	17·1	18·2	26·0	16·1
Total soluble-N . . .	694	837	790	853	780	823	627	781	631	719	650	714	629	693

from the bulb to the extent of 2 to 3 inches. The bulbs which had been planted closely in flats, each containing sixty bulbs as in commercial practice, were then transferred to sections of the greenhouse in which the minimum temperatures were regulated at 40° F., 50° F., 60° F., 70° F. During exposure to these different temperature conditions the plants were subjected to approximately 12 hours of day and night, by shading with black cloth. The light conditions during the experiment were those of normal daylight.

The first samples showing the effect of the temperature treatment during growth were taken after 13 days of treatment when the plants at 70° F. were approaching the mature condition of the bloom. In order to establish that the differences observed at 13 days were not due to differing stages of plant maturity, rather than to the temperature treatment, some plants grown at the lower temperatures were allowed to proceed to an equivalent stage of maturity. This occurred at 20 days for the plants at 60° F., 37 days for the plants at 50° F., and 52 days for the plants at 40° F. Samples were taken at each sampling period at the end of a dark period and at the end of an ensuing light period of about 9 hours. The data which resulted from this experiment are recorded first in Table II, where each amino-acid is recorded as micrograms of α-amino-nitrogen per gram fresh weight of leaf, and ammonia and total soluble nitrogen are also recorded as micrograms nitrogen per gram fresh weight. In Fig. 1 the effects of temperature during growth and of diurnal variation, as shown by the comparison of the light and dark samples, are shown for the major nitrogenous constituents. The main conclusions arising from the data of Table II and Fig. 1 are as follows:

First, it will be evident that the effects ascribed to the temperature conditions during growth could not be due to differences in the stage of maturity of the plants, since plants grown at 40° F., whether harvested after 52 or 13 days of growth, have similar composition.

Little information concerning the effects of these experimental treatments can be drawn from the values of the total soluble nitrogen since these tend to reflect, in large measure, the contribution of the major constituent, namely, γ-methyleneglutamine. Moreover, this constituent changed, relatively, much less over the whole range of treatments than did the other substances that will be considered, although the changes in the absolute amounts were in some cases high in comparison with the changes in the other substances present at a lower level. Transfer from dark to light conditions did appear to increase the content of γ-methyleneglutamine in the tulip leaves—particularly where the plants were grown at the lowest temperatures of 40° F. In this case the increase of γ-methyleneglutamine in the light period approached 40 per cent. When the plants were grown at the higher temperature (70° F.) and the leaves had a higher content of γ-methyleneglutamine, even in the dark, the additional effect of light was not noticeable.

Proceeding now to the effects of the treatments on the detailed behaviour of other nitrogen compounds, the effects will be most easily discerned by reference to Fig. 1.

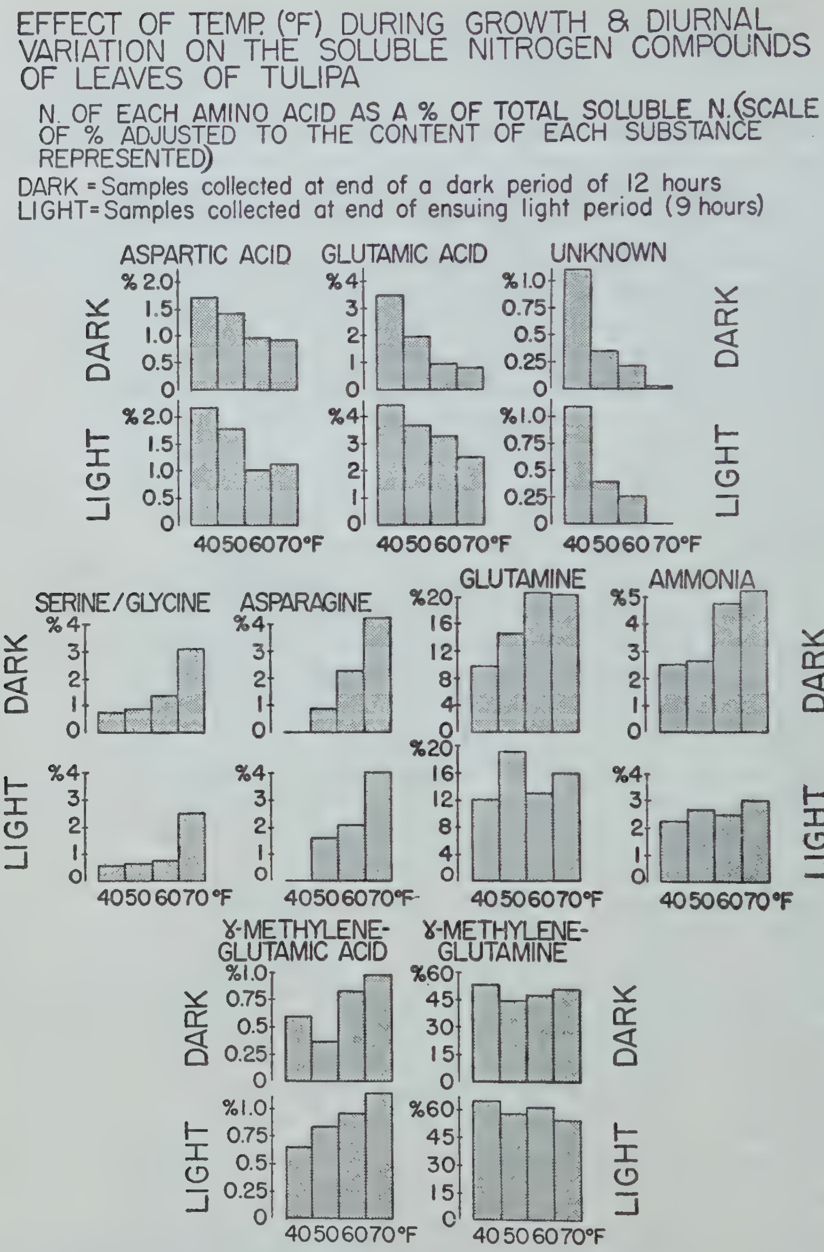

FIG. 1. Effect of temperature (°F.) during growth, and diurnal variation, on the soluble nitrogen compounds of leaves of *Tulipa*.

N of each amino-acid as a per cent. of total soluble nitrogen.

Scale of per cent. adjusted to the content of each substance represented.

Dark samples collected at end of a dark period of 12 hours.

Light samples collected at end of ensuing light period (9 hours).

Although γ-methyleneglutamic acid is invariably a minor constituent compared to γ-methyleneglutamine, never reaching much more than 1 per cent. of the total soluble nitrogen, nevertheless it does seem to be responsive to the temperature conditions that prevail during growth, for the content in the plants from the higher temperatures is greater than that in the plants from the lowest temperatures, irrespective of the light or dark conditions at the time of sampling.

Certain groups of amino-acids tend to vary in a similar fashion in response to the temperature treatments during growth. One such group consists of aspartic acid, glutamic acid, and the unknown substance U131. The contribution of these substances to the total soluble nitrogen tends to decrease at the higher temperatures during growth. This was true whether the plants were sampled after a dark or a light period. In addition the dicarboxylic acids, aspartic acid and glutamic acid, tended to be present in larger amounts when the plants were sampled after a light rather than after a dark period.

Another group of amino-acids tended to increase as a percentage of the total soluble nitrogen when the growth temperature was increased. These are serine and glycine considered as a unit, asparagine, glutamine, and free ammonia. This trend with temperature tended to be evident both for plants sampled after a dark and a light period, though it was not so apparent in the case of glutamine and free ammonia when the plants were sampled at the end of a light period.

There is an obvious reciprocal relationship between the relative content of asparagine and the unknown substance designated U131. The plants grown at the lowest temperatures had virtually no asparagine whereas they had a relatively high content of U131, and the plants grown at the highest temperatures had a relatively high content of asparagine but virtually no U131. Thus it was possible, merely from the qualitative appearance of the chromatograms, to assign plants correctly to the conditions under which they had been grown.

Changes in the soluble nitrogen compounds of leaves of Tulipa *of excised shoots maintained in light and dark*

A feature of the results described has been the relative stability of the γ-methyleneglutamine content in the leaves of the tulip, despite a wide range of treatments during growth. Attention was next turned to the possible effects that might be induced by exposing excised shoots to continuous light and darkness, recognizing that in excised monocotyledonous leaves, notably barley, protein breakdown occurs rapidly in the dark and somewhat less so in the light, with consequential effects on the composition of the soluble nitrogen.

For these experiments plants were obtained at the stage at which the floral shoot was still immature and this was removed so that the shoots, excised at their base, had two expanded leaves. The excised shoots were placed in distilled water, which was frequently changed in the course of the experiment, and exposed to continuous light or darkness in a room regulated at 72–75° F. Samples were taken and subjected to the usual extraction and analytical procedures at the beginning of the experiment and after 3, 7, 10, and 14 days of treatment in both the light and the dark. After 14 days of these treatments, senescence and breakdown in the leaves of the excised shoots was apparent, as shown by the yellowing of the leaves, and this was accentuated in the dark. The data which accrue from this experiment are shown in Table III in which each amino-acid is recorded as micrograms of α-amino-nitrogen per gram fresh weight of leaves, and ammonia, soluble nitrogen and total nitrogen as micrograms of nitrogen per gram fresh weight of leaves. Fig. 2 shows the effect of treatments on protein breakdown as shown by the ratio of soluble to protein nitrogen in leaves and also traces the changes in those constituents most affected by the treatments.

TABLE III

Shows the variation in amounts of various nitrogenous compounds present in *Tulipa* leaves, which were excised and maintained in water for differing periods of time in continuous darkness and in continuous light. Data are expressed as micrograms α-amino-N per gram fresh weight of leaf material for the amino-acids, and as micrograms N per gram fresh weight for ammonia, total soluble and total nitrogen. Each figure documented is a mean of determinations on two separate samples.

	Dark					Light				
	0 days	3 days	7 days	10 days	14 days	0 days	3 days	7 days	10 days	14 days
Aspartic acid	6·3	5·8	16·9	21·1	10·4	6·3	4·9	13·8	12·8	8·0
Glutamic acid	10·7	11·6	12·5	12·5	10·5	10·7	11·2	12·0	10·6	6·0
Serine/glycine	11·2	13·4	40·5	59·5	55·2	11·2	28·1	31·9	22·3	10·1
Asparagine	12·1	13·7	36·5	75·7	86·1	12·1	20·0	32·8	24·0	20·7
γ-methylene-glutamic acid	4·4	1·9	2·4	8·4	12·4	4·4	2·1	7·8	6·1	7·1
Threonine	1·8	1·6	3·5	3·3	3·9	1·8	4·6	3·2	1·7	0·7
Alanine	5·8	9·9	42·4	63·1	75·6	5·8	30·4	25·2	12·8	9·5
Glutamine	26·7	90·6	210	243	248	26·7	79·8	196	136	108
γ-methylene-glutamine	155	159	157	163	152	155	159	166	177	168
Lysine	1·2	4·8	6·2	3·4	1·9	1·2	4·9	6·2	2·6	2·2
Arginine	1·6	4·8	6·5	4·4	4·4	1·6	4·1	6·3	3·0	3·1
Valine	1·3	5·4	7·2	7·4	16·6	1·3	5·3	9·4	2·0	2·8
Leucines	1·7	3·5	13·8	8·1	12·6	1·7	11·3	11·8	1·3	trace
Tyrosine	trace	4·1	6·8	4·2	7·4	trace	3·6	6·6	2·2	2·2
Ammonia-N	17·2	22·5	38·3	48·4	73·9	17·2	19·5	22·8	27·7	32·9
Soluble-N	536	708	1,127	1,367	1,478	536	844	1,065	941	832
Total-N	2,630	2,610	2,590	2,490	2,190	2,630	2,431	2,220	2,000	1,751

The data in Table III indicate that protein breakdown occurred in the excised shoot both in the light and in the dark at approximately the same rate, at least until the end of the seventh day. Subsequently the proteolysis continued in the dark until the end of the experiment, whereas in the light it seemed to be arrested. In fact the content of various amino-acids actually tended to decrease in the light toward the end of the experiment, though this was correlated with some disappearance of soluble and total nitrogen from the leaves, a disappearance which presumably occurred to the external solution.

An outstanding result is that the content of γ-methyleneglutamine in these

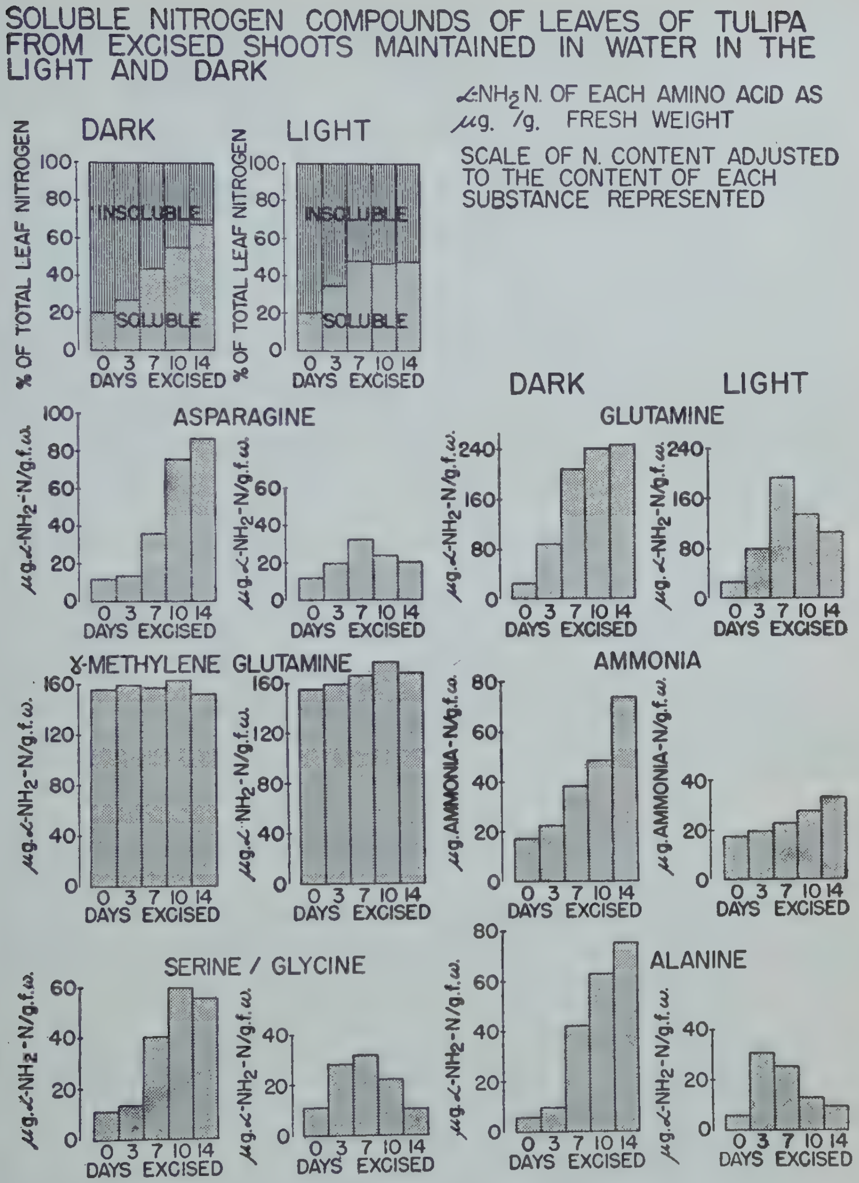

FIG. 2. Soluble nitrogen compounds of leaves of *Tulipa* from excised shoots maintained in water in the light and dark.

$\alpha.NH_2.N$ of each amino-acid as µg./g. fresh weight.

Scale of N content adjusted to the content of each substance represented.

leaves remained remarkably constant throughout the entire range of treatments in both the light and the dark. The range of variation in the content of γ-methyleneglutamine was less than 20 per cent. This uniformity in the content of γ-methyleneglutamine was in respect of the absolute amounts present; its contribution to the total soluble nitrogen tended to decrease because the other constituents increased as a result of protein breakdown. As protein breakdown proceeded the content of free ammonia in the leaves also increased—as expected this increase was greater in the dark than in the light.

In tulip leaves in the dark the glutamine content increased very rapidly, almost tenfold in a period of 14 days. The asparagine content of the same leaves in the dark was consistently at a lower level although the relative increase was again quite marked, approximately sevenfold in 14 days. The increase in asparagine plus glutamine of the excised shoots tended to be at approximately the same rate in the light, up to 7 days, as in the dark. Thereafter in the light these amides behaved like other soluble constituents and tended partially to disappear from the leaves. In leaves which were exposed to darkness even those amino-acids which were not always initially present in determinable amounts, such as arginine, tended to increase as proteolysis occurred.

The overall conclusion of these experiments is that the excised shoots of tulip show the typical behaviour of monocotyledonous leaves exposed to continuous darkness or light with the added fact that the γ-methyleneglutamine is present as a relatively inert constituent, surprisingly unaffected by this range of environmental conditions. It might well be that the use of another species of tulip in which the γ-methyleneglutamine is present in much smaller amount might yield evidence that this substance can be affected by this range of experimental conditions.

The incorporation of C^{14} from $C^{14}O_2$ into the alcohol-soluble compounds of leaves of excised shoots of Tulipa

Excised shoots were placed in water and exposed to continuous light at 24° C. 0·15 millicuries of carbon as $C^{14}O_2$ were supplied to 20 g. of leaves. After 60 hours virtually complete absorption occurred.

The leaf material was then extracted with ethanol in the usual way except that a part of the material, as macerated in alcohol, was treated with acid-ethanolic dinitrophenylhydrazine to fix the keto acid components as their hydrazones, after the method used for determining keto acids (Towers, Thompson, and Steward, 1954). The nitrogen compounds from the remaining portion of the macerate in ethanol were then completely extracted in the cold and the alcohol extracts concentrated and chromatograms of a suitable aliquot were made. Duplicate chromatograms were used for the quantitative determinations and for the radioautographs. The radioautographs were made by exposing Ansco X-ray no-screen film to the papers for a period of approximately 4 weeks. Radioautographs from chromatograms of protein hydrolysates were also made. From these operations qualitative observations were made on the distribution of C^{14} in the soluble compounds and data were obtained which permitted the specific activity of selected nitrogen compounds to be expressed as counts per minute per milligram of amino-nitrogen in the substances in question.

After 60 hours of contact with leaves on excised shoots as many as fifty different compounds were labelled but most of the C^{14} absorbed from $C^{14}O_2$ was in the sugars, glucose, fructose, and sucrose. Since but little protein synthesis occurred under these conditions it is not surprising that only a few amino-acids (aspartic acid, glutamic acid, serine, glycine, alanine, and arginine) in the protein hydrolysate were found to have received C^{14} and even in these the specific activity was small in comparison with the soluble compounds, especially the sugars. The free amino-acids which were most readily accessible to the $C^{14}O_2$ were aspartic acid, glutamic acid, glutamine, γ-methyleneglutamine, γ-methyleneglutamic acid, and valine. The specific activity (expressed as counts per minute per milligram of α-amino-nitrogen) for glutamine (99,600) was sixteen times that of γ-methyleneglutamine (6,220).

The radioautograph of the chromatogram produced by reduction of the keto acid dinitrophenylhydrazones showed radioactivity in the following substances: γ-methylglutamic acid, alanine, aspartic acid, and twelve unidentified substances, five of which did not react with ninhydrin (Fig. 3). Therefore, the $C^{14}O_2$ went into pyruvate and oxaloacetate. The marking of γ-methylglutamic acid, though consistent with the marking of α-keto-γ-methyleneglutaric acid, is not proof of this because α-keto-γ-methylglutaric acid would give the same product. A radioactive spot in the position occupied by γ-hydroxy-γ-methylglutamic acid indicates that its keto-acid analogue, which has been detected elsewhere (Grobbelaar, Pollard, and Steward, 1955; Virtanen and Berg, 1955), occurs in tulip and may have become radioactive. On the other hand no evidence of free α-ketoglutarate appears.

The outstanding result of this experiment is the comparative slowness with which the γ-methylene compounds received C^{14} from $C^{14}O_2$ even though the activity spread from the relatively strongly marked sugars through a large number of known and unknown carbon compounds. For example, glutamine reached a very much greater specific activity than did the γ-methyleneglutamine, suggesting that the amination of α-ketoglutarate and the amidation of glutamic acid can readily occur even in the excised shoot.

A comparative failure of the tracer carbon to enter the pool of a given nitrogen compound (γ-methyleneglutamine) from clearly labelled sugars and/or its appropriate keto acid implies that the compound in question was not, at this stage of the development of the excised shoot, rapidly turning over. This could be due either to sluggish utilization of this material or to some lack in the mechanism of its formation, namely, the amination of its keto analogue. This evidence, therefore, indicates that the amination of the carbon skeleton either does not occur in the adult leaves, when detached from the root, or alternatively synthesis may only occur in the young growing leaf.

Whereas glutamine and asparagine bear, in the tulip, conventional relations to protein metabolism, γ-methyleneglutamine does not do so. This would seem to indicate that its metabolism is somewhat remote from the more normal routes of nitrogen metabolism and its formation represents a superimposed and independent pathway of nitrogen utilization. Thus, the main difference of *Tulipa* from other Liliaceous plants may be in the possession of this separate phase of nitrogen metabolism.

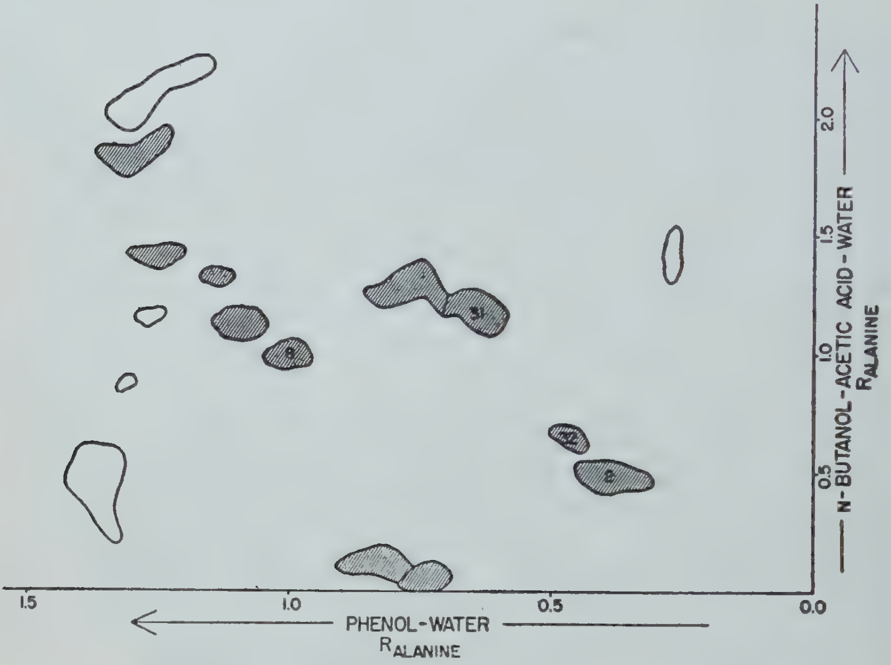

FIG. 3. Map of positions on phenol: butanol-acetic acid chromatograms of C^{14} labelled substances derived from acidic, ethyl acetate soluble compounds occurring free in leaves of *Tulipa gesneriana.*

▨ Ninhydrin reactive substances derived from carbonyl compounds by conversion to amino compounds.

○ Substances which do not react with ninhydrin.

Key: 2, aspartic acid; 8, alanine; 31, γ-methylglutamic acid; 32, γ-hydroxy-γ-methylglutamic acid. Other ninhydrin reactive substances not yet positively identified.

SUMMARY

1. The free amino-acids present in fourteen species of *Tulipa* leaves have been quantitatively assayed. γ-methyleneglutamine constitutes from 0–70 per cent. of the total nitrogen present in the free amino-acids, reaching very high absolute concentrations in some species, e.g. about 2 g. per kilogram of fresh *Tulipa gesneriana* leaves. Its occurrence in *Tulipa* then appears as a feature superimposed upon an otherwise normal complement of amino-acids and may represent a biochemical-genetic characteristic developed during breeding and selection within the genus.

2. The effects of temperature during growth of *Tulipa gesneriana* produced noticeable differences in the amino-acid composition of the leaves. Aspartic acid, glutamic acid, and an unidentified substance (U131) tended to decrease

in absolute and relative amounts at the higher growth temperatures, whereas serine/glycine, asparagine, glutamine, γ-methyleneglutamic acid, and ammonia tended to increase. Whilst temperature affected the absolute amounts of γ-methyleneglutamine in the leaves to an extent equal to, or greater than, most other amino-acids, the relative changes were small due to the large amounts present.

3. Plants sampled at the end of the light period (9 hours) had recognizably different leaf compositions from those sampled after a dark period (12 hours). Aspartic and glutamic acid concentrations tended to be greater in plants from the light than in those from the dark, irrespective of the temperature during growth. γ-methyleneglutamine concentrations were 40 per cent. greater in plants taken from the light than in plants taken from the dark at 40° F. Higher temperatures during growth produced smaller differences in the γ-methyleneglutamine contents.

4. Excised tulip shoots were maintained in continuous light or darkness for 14 days, and the nitrogenous composition of the leaves was determined at intervals. γ-methyleneglutamine concentrations changed by less than 20 per cent. during the whole time, although protein breakdown was occurring rapidly in the leaves. The amides and other amino-acids tended to increase. Glutamine concentrations increased nearly tenfold in the dark, and asparagine concentrations sevenfold. Free ammonia also markedly increased. After 7 days, further protein breakdown in the leaves maintained in the light was much slower than in the leaves in the dark. In these respects tulip leaves resemble other starved monocotyledonous leaves.

5. C^{14} and $C^{14}O_2$ readily enters a wide range of compounds in illuminated, excised shoots of *Tulipa*. However, C^{14} entered amino-acids relatively slowly, especially γ-methyleneglutamine which was present in large amounts. The specific activity of γ-methyleneglutamine was only one-sixteenth of that of glutamine. This suggests that, in the *excised, mature* leaf, synthesis of nitrogen compounds from sugars (via keto acids) was limited, probably at the amination stage since radioactivity occurred in certain keto acids.

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Dr. John K. Pollard assisted at various points but especially in cases where γ-hydroxy-γ-methylglutamic acid or γ-hydroxyglutamic acid were concerned.

The investigation was also aided by facilities and assistance made possible by the grant from the Grasselli Chemicals Department of the E. I. du Pont de Nemours & Company, Inc. to Cornell University for work being directed by one of us (F. C. S.). For all this help we express our thanks.

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Experimental and Analytical Studies of Pteridophytes

XXXV. The Effects of Direct Applications of Various Substances to the Shoot Apex of *Dryopteris austriaca* (*D. aristata*)

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With Plate IV and seventy-three Figures in the Text

ABSTRACT

The organization and morphogenetic activity of a fern apex, that of *Dryopteris austriaca* (*D. aristata*), have been further investigated by observing how it responds to direct applications of solutions of various physiologically active organic substances. Large apices were laid bare and treated for periods of up to about 20 days with solutions of 3-indoleacetonitrile, dinitrophenol, maleic hydrazide, yeast extract, glutamine, &c., with direct examination at frequent intervals using a binocular microscope.

In most cases some reaction was obtained. Developments of morphogenetic interest include: an increase in the rate of growth in the subapical region; an arrest or slowing down of growth in the apical meristem; the formation of flattened and of sunken, cup-shaped apices; the formation of double, treble, and quadruple leaf primordia, of two primordia in a single leaf site, and of centric (or radial) leaves; the occasional suppression of primordia; the reversal of the phyllotactic spiral; the induction of buds on meristems with an uninjured apical cell; the promotion of root formation and growth; the formation of scales on the apices of young leaf primordia and leaf sites, and the precocious formation of scales over the whole of the apical meristem, including the apical cell; and the precocious parenchymatization of the meristem including the apical cell. The significance of these results is discussed.

I. INTRODUCTION

IN the investigations now described, an attempt has been made to observe what effects are induced in a fern apex when solutions of various physiologically active substances are directly applied to it. Some work along these lines, using flowering plants, has already been described by M. and R. Snow (1937) and Ball (1948), and for ferns there is the work of Allsopp (1952, 1953, and 1956). A considerable contemporary literature deals with various abnormal morphological developments induced by applications of growth-regulating substances, herbicides, &c., to the plant as a whole (Gavaudau, Debraux, and Antigny, 1951; Gavaudau and Debraux, 1952; Gorter, 1951; Linser *et al.*, 1955; Thaler, 1954, &c.). A special feature of the present work is that the apex used, that of *Dryopteris austriaca* (Jacq.) Woynar (*D. aristata* Druce), has not only been investigated previously in some detail by anatomical and surgical techniques but, as it is of large size and possesses considerable viability under experimental treatment, it can readily be inspected from day to day, and thus enables any unusual or transient organizational features or

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morphogenetic developments to be detected at the outset and followed up over a considerable period of time.

II. THE PROBLEM OF ORGANIZATION

Tentative views on the organization of different regions of the apical meristem have already been expressed by Schoute (1913, 1936), Richards (1948), and Wardlaw (1951*a*, *b*; 1955*b*). These need not be reviewed in detail but, as a working hypothesis based on them, the writer now proposes to regard the shoot apical meristem as a complex physico-chemical reaction system (or a group of contiguous and interrelated reaction systems) which, during its normal functioning, gives rise on its margin to regularly spaced, superficial growth centres, or loci of special metabolism, which usually become leaves, and to an associated tissue pattern within. In the present investigation the aim is to interfere with the normal functioning of this apical reaction system by chemical means, using a series of substances which are known to have a special influence on growth and metabolism. The general problems involved are discussed by Bünning (1952), Skoog (1954), and Wardlaw (1952). It might be possible to interfere with the apical reaction system so as to effect changes in its normal chemical and morphogenetic pattern. Thus the present investigation was to see if, by direct chemical treatment of the apex, (*a*) the characteristic organization of the meristem, including the regular inception of new growth centres, and (*b*) the nature and further development of individual growth centres (which usually become leaves, but may be caused to develop into buds by appropriate surgical treatments), could be modified.

III. MATERIALS AND METHODS

Large apices of *Dryopteris austriaca* (*D. aristata*), on pieces of rhizome about 3 cm. in length, were laid bare as already described (Wardlaw, 1947), all the older leaf primordia and scales being removed. The intact conical apical meristem, surrounded by the seven to ten youngest primordia, was then ready for chemical treatment. This consisted in soaking small pads of absorbent cotton-wool in the prepared physiological solution and placing them gently on the apices, the pads being usually renewed two or three times a week. In this way a thin film of solution was more or less consistently maintained over the whole of the apex during the period of treatment. The rhizome pieces were grown in moist peat in flat pots covered by a sheet of glass. The apices were examined once or twice weekly, depending on the rate of growth, under a binocular microscope. In the several experiments, the apices thus treated—and for convenience here described as *intact apices*—were usually accompanied by other, comparable apices in which the apical meristem had been isolated from the subapical region by four deep vertical incisions, as described in an earlier investigation (Wardlaw, 1947). In some of these apices, the incisions were made so as to include the five youngest primordia, (P_1–P_5), on the isolated central plug; in others the incisions were made higher up on the apex so as to exclude these primordia. All of these are here referred to as *isolated* *apices*. By applying solutions to incised apices it was hoped that it might be possible to detect primary effects in one or other, or both, of the apical and subapical regions.

In each of the initial experiments, two or three intact apices and the same number of isolated apices were given chemical treatment, while other prepared but untreated apices from the same batch or collection of material served as controls. As soon as a treatment yielded, or seemed likely to yield, results of interest, the experiment was repeated using further batches of materials. This also afforded some information on the seasonal reactivity of apices. During 1955 some nine batches of apices were treated experimentally between May and August, and in all a very considerable number of apices—approximately 180—came under close observation. Some experiments along the same lines had also been undertaken in 1952 and 1954.

Table I gives a list of the substances used in these experiments and an indication of the principal developments observed. The concentrations used were mainly based on indications given in the literature, e.g. yeast extract at 500 mg. per litre is known to promote active growth in aseptic cultures of apices of *Adiantum pedatum* (Wetmore, 1949, 1953, 1954). In general, since a thick cuticle covers the apical meristem of *Dryopteris*, somewhat high concentrations were used in the initial experiments, e.g. those of dinitrophenol and maleic hydrazide are known to kill the delicate apices of *Marsilea Drummondii* grown in aqueous culture (Allsopp, 1955). Information as to suitable solutions used in tissue culture is reviewed by Gautheret (1955), and for the inhibition of respiratory activity by James (1953).

The terminology used here is that which is now generally adopted in the literature of phyllotaxis: P_1, P_2, P_3, &c., indicate the young leaf primordia round the apical meristem in the order of increasing age; at the time of operation or exposure, I_1 indicates the site of the next (but as yet invisible) leaf primordium to be formed, and I_2 the next after that, and so on; but I_1, I_2, &c., are also used to denote the primordia which eventually develop in these respective sites. The term 'apex' is used in a general way to indicate the distal region of the shoot and includes the apical meristem and subapical region.

IV. EXPERIMENTAL OBSERVATIONS

(*a*) *General survey of results*

When a solution is applied to the apex as described in Section III, it may be taken in by any of the superficial tissues, from the apical cell downwards towards the region of maturation; or it may be taken up by some regions but not by others; or it may not be taken up at any point. The entry of some substances by way of wounds may be important; localized or more general effects may be thereby induced, or the effects may be transitory. *A priori*, the entry of a solute may cause (*a*) a general inhibition of growth and of organ formation and development in the apical and subapical regions, or (*b*) a greater retardation in the apical meristem than in the subapical region (the reverse effect is regarded as improbable), or (*c*) a greater acceleration of growth in one of these regions. In (*b*) and (*c*) secondary effects may be induced in the apical meristem as a result of the increased relative growth in the subapical region, e.g. metabolites may be withdrawn from the cells of the distal region and its embryonic or meristematic character may be modified, and organs developing in the region of transition may be subjected to tangential stress. The observations set out in Table I show that several of the chemical treatments have yielded results of morphogenetic interest along the lines indicated above. In particular, it will be noted that the application of substances of very different physiological activity has led to closely comparable morphological developments, e.g. the formation of double leaves, a scaly development over the apical meristem, &c.

Past experience has shown that the reactivity of the apex of *Dryopteris* to experimental treatment may vary considerably according to the season (Wardlaw and Cutter, 1956). This has also been found in the present series of experiments, e.g. apices collected in June differed in their response to a particular treatment from those collected earlier or later in the year. The state of the apical reaction system is thus seen to be important. It is recognized that this variability is almost certain to make the interpretation of results more difficult. In this investigation, however, the aim was to ascertain whether or not the apex of *Dryopteris* would show any considerable measure of reactivity to a variety of chemical treatments. The records now presented demonstrate that the normal activity of the apex can be modified in a number of ways that are of direct interest in the investigation of organization and morphogenesis.

(*b*) *Effects of 3-indoleacetonitrile* (*IAN*)

This is a naturally occurring plant hormone (Jones *et al.*, 1952).

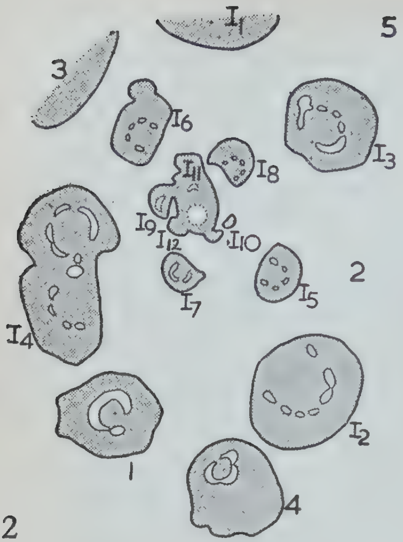
Apices from five different batches were kept under observation after treatment with IAN, concentrations of 1, 10, 100, and 1,000 mg. per litre being used. The period of treatment ranged from 10 to 24 days, observations being continued for a considerable time thereafter. At the highest concentration the apical meristem, the surrounding primordia, and the tissues of the subapical region were killed and blackened. At the two lower concentrations no developments of special morphogenetic interest were noted. But, in apices treated with 100 mg./l. IAN, there was a rapid acceleration of growth in the subapical region and in the older leaf primordia borne upon it. As a result the apical cone soon came to occupy a position at the base of a cup-like depression, the sunken meristem being closely comparable with that typically found in the adult shoots of certain other ferns (R. and C. Wetter, 1954; Wardlaw, 1956). There was also evidence of an initial arrest or retardation in the growth of the apical meristem, but further work would be necessary to establish this point definitively. These sunken apices were very subject to mechanical injury. (As the I_1 position is usually indicated in these experimental materials by removing a tangential panel of tissue on the abaxial side of P_8, thereby leaving a gap or weak area in the aforementioned enlarging rim, a further effect of this

TABLE I

Chemical Treatments of Apices of Dryopteris

+ indicates evidence of a positive reaction to the treatment

Substance	Concentration	Some reaction	Arrest of growth in meristem	Abnormal enlargement of subapical region	Arrest of leaf formation or development	Precocious scaly development of apices of primordia	Precocious scaly development of apical meristem	Formation of double leaves	Induction of buds on intact meristem	Increased root formation
Controls	..	..	..	..	..	..	..	..	..	..
Potassium nitrate	200 mg./l.	..	..	..	..	..	..	..	..	..
Glutamine	500 mg./l.	+	?+	..	..	+	+	+	..	..
Adenine sulphate	200 mg./l.	?+	..	..	..	..	..	..	..	..
Adenosine triphosphate	200 mg./l.	+	+	..	+	+	..	..	..	..
Desoxypentose nucleic acid	1,000 mg./l.	+	..	+	+	..	..	..	..	..
3-Indoleacetic acid	1,000 mg./l.	+	?+	+	..	..	..	+	..	+
,,	100 mg./l.	+	?+	+	..	+	..	+	..	+
,,	10 mg./l.	—	..	..	..	..	..	..	..	..
,,	1 mg./l.	—	..	..	..	..	..	..	..	..
3-Indoleacetonitrile	1,000 mg./l.	+	+	+	..	..	..	..	..	..
,,	100 mg./l.	+	?+	+	..	..	..	+	..	..
,,	10 mg./l.	—	..	..	..	..	..	..	..	..
,,	1 mg./l.	—	..	..	..	..	..	..	..	..
Yeast extract	500 mg./l.	+	+	+	+	+	+	+	+	..
Thiamine hydrochloride	100 mg./l.	+	+	+	+	+	+	+	+	..
Coconut milk	Full strength autoclaved	+	+	..	..	..	..	..	..	..
2, 3, 5-Triiodobenzoic acid	200 mg./l.	+	+	..	+	..	+	..	+	..
2, 3, 6-Trichlorobenzoic acid	20 mg./l.	+	+	..	+	..	..	..	..	..
Sodium arsenite	0·1 m.M.	+	+	..	+	..	..	+	..	..
Coumarin	100 mg./l.	+	+	..	+	+	+	+	..	..
Maleic hydrazide	100 mg./l.	+	+	..	+	+	+	+	+	..
Dinitrophenol	0·1 m.M.	+	+	..	+	+	+	+	+	..



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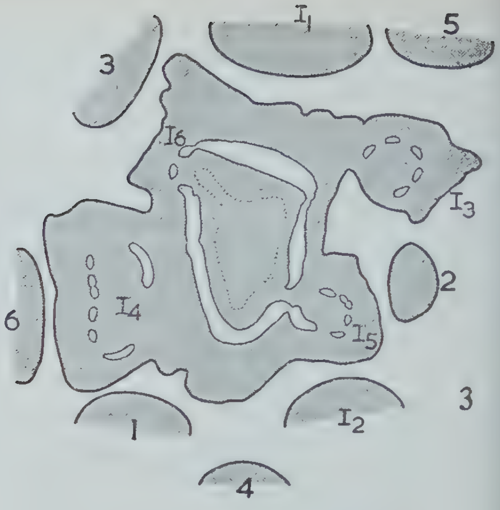

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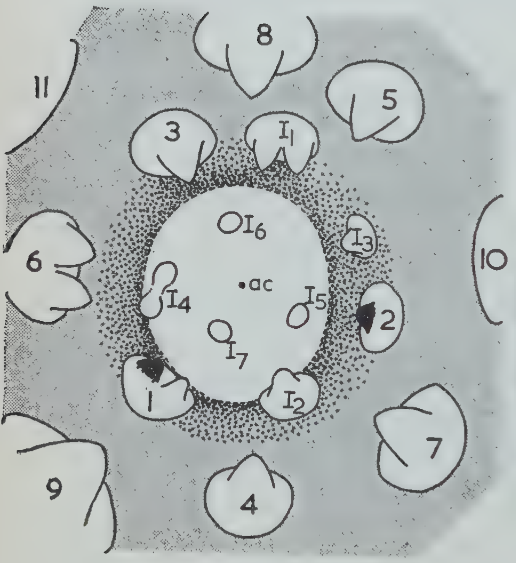

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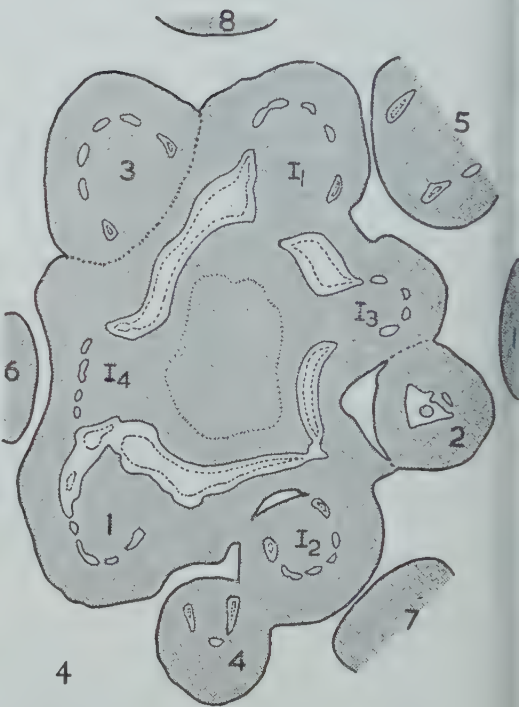

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FIG. 1. Downward view of an apex, as seen under the binocular microscope, after treatment with 3-indoleacetontrile. The apical cone (the oval, light central area) with the apical cell (*ac*) at its tip, is seated in a cup-shaped depression, due to the rapid growth of the subapical region (stippled) and older primordia, P_1–P_{11}. Since the beginning of the experiment, new primordia I_1–I_7 have appeared, with a reversal of the phyllotactic spiral at I_3. I_1, P_1, P_6 are tangentially extended double primordia; I_4 which has just appeared is seen to consist of two contiguous primordia, arising in a single leaf site. The tips of P_1 and P_2 have been mechanically injured (× 13). FIGS. 2–4. The same apex as in Fig. 1, cut transversely, and seen at different levels (× 10). FIG. 2. Close to the tip of the apex: new primordia up to I_{12} have been formed; the double nature of I_4 is clearly shown; I_{10} is seen to be a double primordium. FIG. 3. Somewhat lower down than Fig. 2: showing the reversal of phyllotaxis and the tangential enlargement of I_4. FIG. 4. Still lower down, showing the broad leaf-trace and leaf-gap of I_4, but otherwise an approximately normal cross-sectional pattern.

growth enlargement was to extend the apex somewhat in the axis of I_1–P_4 and the apical cone became oval in outline (Text-fig. 1).

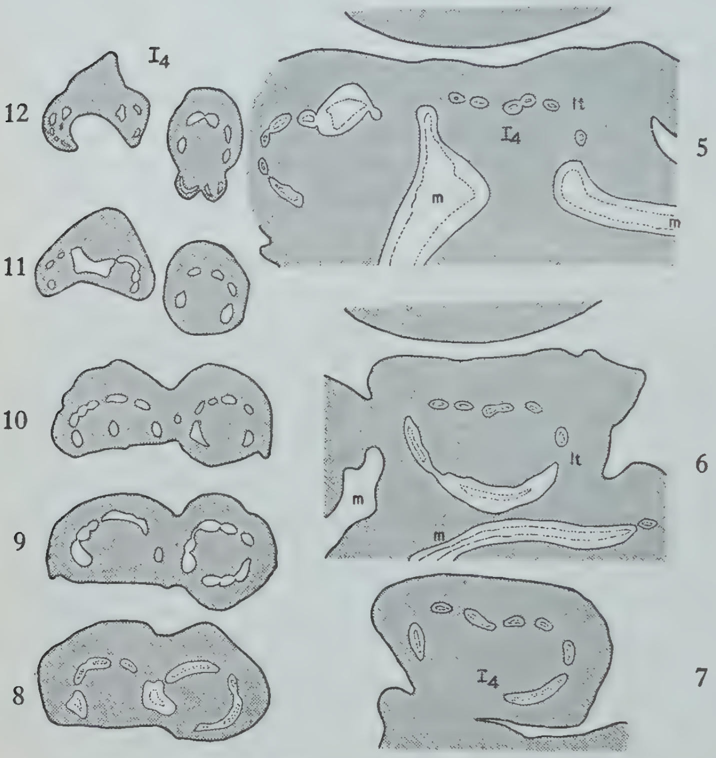
The reaction of a representative apex may now be considered in greater detail (Text-fig. 1). This apex was prepared on 16 May 1955, treatment with IAN being maintained for 20 days and observations continued for 67 days, when the apex was fixed for sectioning. Soon after the initial treatment, P_1 and P_6 were seen to be developing as bifurcated primordia. Also, although the initial formation of I_1 was normal, i.e. an elliptical mound, two growing points were established at an early stage and a double primordium was formed. On the first appearance of I_4, the site was seen to be occupied by two contiguous outgrowths, so that the resulting primordium was double from its inception. During the further growth of this specimen, with the emergence of new primordia up to I_{12}, there was a gradual diminution in the size of the apical meristem. The conformation of the later foliar members was ascertained from transverse serial microtome sections, some of which are illustrated in Text-figs. 2–4.

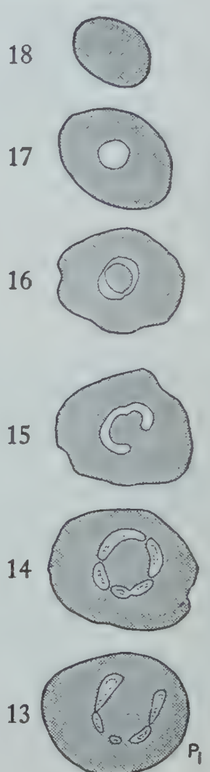
As Text-fig. 2 shows, I_4 was greatly enlarged tangentially. Other double primordia were also abnormally large. It is of interest that a reversal of the phyllotactic spiral had taken place about the time of formation of I_3 and I_4, but thereafter the sequence of leaf inception was normal. Double leaves were formed in the sites of I_{10} and I_{12} (see also Text-figs. 23, 24). Text-figs. 2–4, in which the cross-sectional structure of the axis is illustrated at different levels in basipetal sequence, show that I_4 was organized as a double primordium from the outset, the two lobes having a common, extra wide leaf-gap. Primordium I_1, on the other hand, was normal in respect of its basal structure and leaf-gap and only became divided some time after its inception. So also in the case of primordium P_1, one lobe of which was damaged during one of the earlier inspections. The cup-like conformation of the apex was well seen in longitudinal sections (Pl. IV, Fig. 6, after treatment with IAA, is quite representative of the IAN effect). This feature became considerably less marked in apices which were allowed to grow on for some time.

Text-figs. 5–12 illustrate diagrammatically the anatomical structure of primordium I_4 from its basal region upwards. Each of the lobes underwent a further dichotomy. Just above the level of insertion of the leaf-trace on the shoot stele, a broad adaxial band of vascular tissue is present; lower down this becomes conjoined with the ribbon-like meristele of the shoot. Below this level the tangentially extended leaf-gap and broad leaf-trace of many strands are conspicuous features.

In this same apex several of the primordia which were present at the beginning of the experiment, e.g. the uninjured half of P_1, P_4, and P_7, developed as radial or centric leaves. These primordia all show the normal dorsiventral symmetry and a horseshoe-shaped leaf-trace in their basal region but, higher up, the primordium became broadly elliptical in cross-section, with radially disposed meristeles (Text-figs. 13–18), a more or less complete solenostele being eventually constituted, Text-fig. 16. Near the distal end of the primordium, this solenostele diminished to a protostele of incipient vascular tissue and finally faded out, the tip of the primordium consisting of parenchymatous tissue only (Text-fig. 18). All the radial primordia were typically of limited growth. In this particular apex, then, both large, fasciated double primordia and small radial ones were present, suggesting that these two developments may, to some extent, be related.

By the time primordia I_{10}, I_{11}, and I_{12} had appeared, the apical meristem had become very small and almost flat. Text-figs. 19–25 show a basipetal series from the shoot apical cell downwards. (These sections dip very slightly between the apical cell and I_{11}: hence, on proceeding downwards, sections through the tip of I_{11} are seen before those of I_{12}, the youngest primordium.) Both I_{10} and I_{12} are double primordia. In I_{12} two slightly separated apical cells could be distinguished, and it is evident that the two contiguous primordia originated in a single leaf site, Text-figs. 23, 24. Had further growth

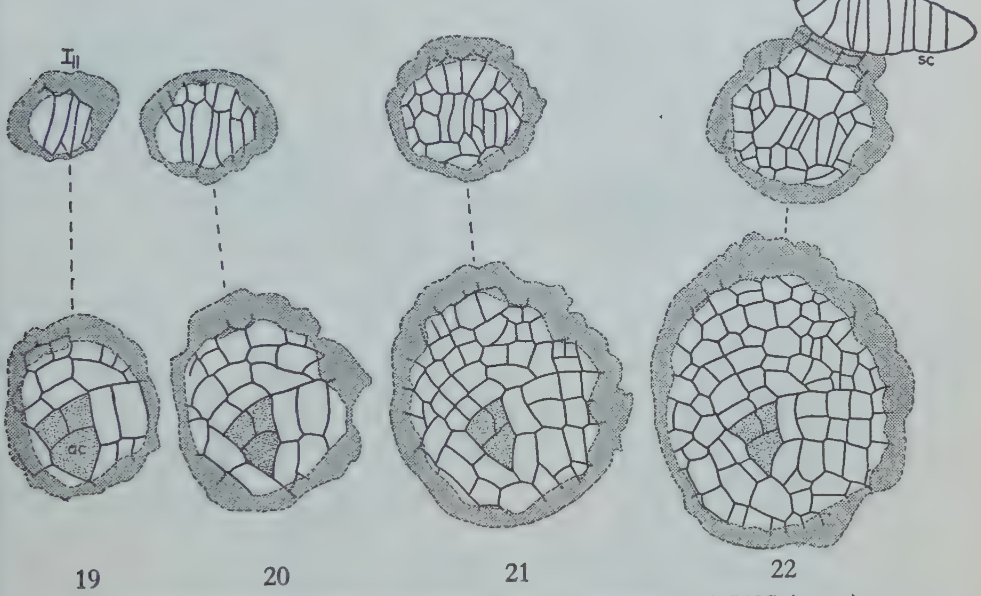




Figs. 5–12. Apex as in Figs. 1–4 (treatment with IAN). These illustrate the cross-sectional outline and vascular development in the double and later quadruple, primordium I_4, from the level of its confluence with the shoot (Fig. 5) upwards to its distal region where the two shanks have undergone dichotomy (Figs. 11–12). *m*, meristele of axis; *lt*, leaf-trace.

Figs. 13–18. From same apex. These illustrate the outline and vascular construction at different levels, from the base upwards, in a radial or centric leaf. (All × 14.)

taken place, the expectation is that I_{12} would have yielded a fasciated primordium very much like I_4. The two primordia, I_{10} and $I_{10'}$, in the I_{10} site (Text-figs. 23–25) probably originated in a similar fashion, but they have become separated tangentially; each shows extended tangential growth, and in neither can the single conspicuous apical cell yet be distinguished (Text-figs. 24, 25). In primordium I_{11}, several apical initials are present whose divisions are characteristically at right angles to the main, i.e. the tangential, component of growth (Text-figs. 19–22).



Figs. 19–22. From same apex as in Figs. 1–4 (treatment with IAN) (×110).

Fig. 19. Transverse section of extreme tip of the apical meristem, traversing the apical cell (*ac*) and the tip of leaf primordium I_{11}. (The stippled region at the periphery indicates the thickness of the section, 10μ.)

Figs. 20, 21. 10μ and 20μ respectively, below Fig. 19; no single initial cell can be distinguished in I_{11}.

Fig. 22. 30μ below Fig. 19, showing the cellular structure of the apex, in which the apical cell can still be distinguished, and of I_{11}. A large scale (*sc*) has already developed close to the latter.

These extended records show that application of IAN not only affected the existing young primordia and those in the course of inception, i.e. P_1 and I_1, but also those which were formed a considerable time after treatment had been discontinued. The records indicate that, although the apical meristem diminished progressively in size, the primordia formed along its basal margin tended to participate in the accentuated development of the subapical region. In those apices in which the apical cell remained intact, buds were not formed.

The observations which follow are based on experiments carried out in 1954 and 1955. When IAN solution (100 mg./l.) was applied to apices which had been isolated by four deep vertical incisions, usually so as to include P_1–P_5 on the plug, a considerable stimulation of growth was again observed in the subapical region. The isolated apices also grew well. Some of these which were fixed after 39 days and sectioned transversely showed that the growth of the apex and the formation of primordia had not been conspicuously retarded. Bud formation in the subapical region—a normal concomitant of this surgical treatment—was not suppressed. These materials have yielded evidence of the enhanced growth of the parenchymatous tissues of the leaf-base and pith, and of the pre-vascular tissue, strongly developed solenosteles being usually observed in the isolated apical plugs.

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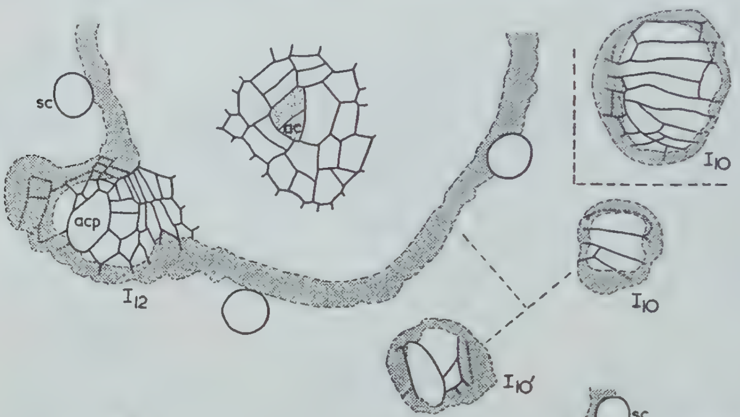

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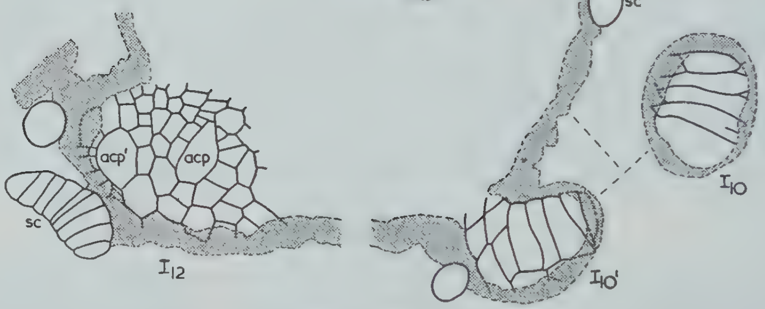
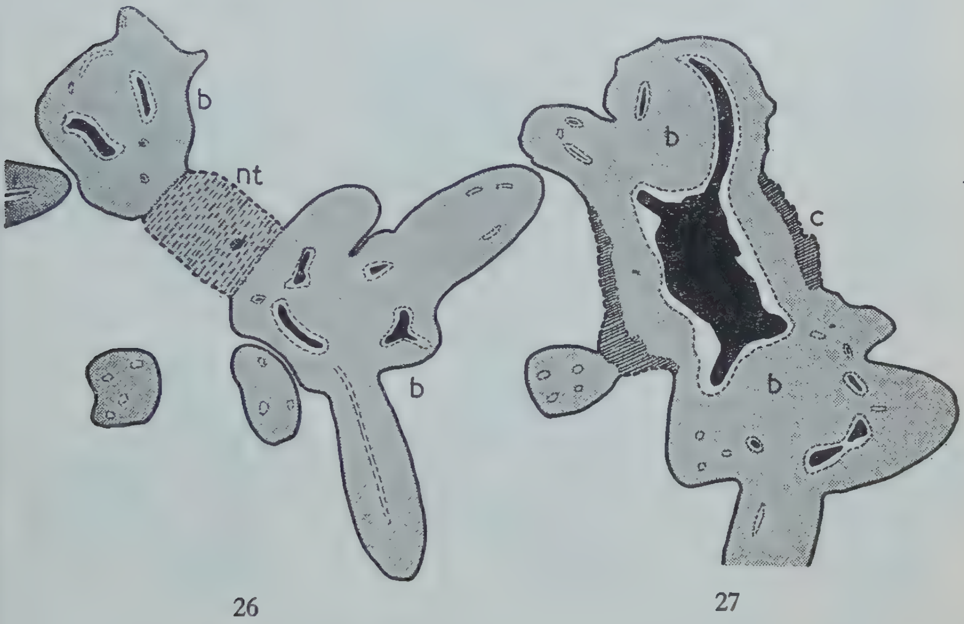


FIGS. 23, 24. Two sections, slightly lower down in the apex than that in Fig. 22, showing the formation of double primordia at I_{10} and I_{12}. At I_{12} the lens-shaped apical cells (*acp, acp'*) of the two contiguous primordia can already be distinguished. At I_{10} the two primordia, I_{10} and $I_{10'}$, have become somewhat separated and also extended tangentially, so that no single apical cell can yet be distinguished.

FIG. 25. Shows primordium I_{10} 10μ lower down than in Fig. 24, showing a tangentially disposed series of superficial cells. (All × 110.)

In June 1952, apices were isolated by four vertical incisions, high up on the meristem, above the level of I_1 and I_2. These incisions were necessarily made relatively shallow, i.e. just penetrating to the pith, as deeper ones at this level almost invariably result in a necrosis of the isolated meristem. IAN in lanoline (1/1,000) was then applied. This concentration is not in itself injurious to the meristem, but, presumably as a result of the incisions, all the isolated meristems became necrosed during the next 5 days. Bud formation soon began in the subjacent region, just outside the incisions, which had also been smeared with the IAN-lanoline. These specimens were eventually fixed after 144 days. The buds were typically large and well developed. In association with each bud there was a strong downward and inward development of vascular tissue which spread into and across the pith of the main shoot. As two or three buds were usually formed at about the same level round the necrosed meristem, the joint effect of this vascular development was seen in the presence of a complete layer of vascular tissue across the pith of the parent shoot. As seen in serial transverse sections of the main axis this vascular development yielded some very curious configurations (Text-figs. 26–27, 28–34, and Pl. IV, Figs. 7–8). On proceeding downwards in the shoot, this medullary vascular tissue faded out and merged with the normal axial vascular system below. Comparable patterns of development were observed in the several specimens from this experiment; in each case the outline of the stelar tissue was in close conformity with that of the axis, a relationship that has already been noted in these experimental studies (Wardlaw, 1947).

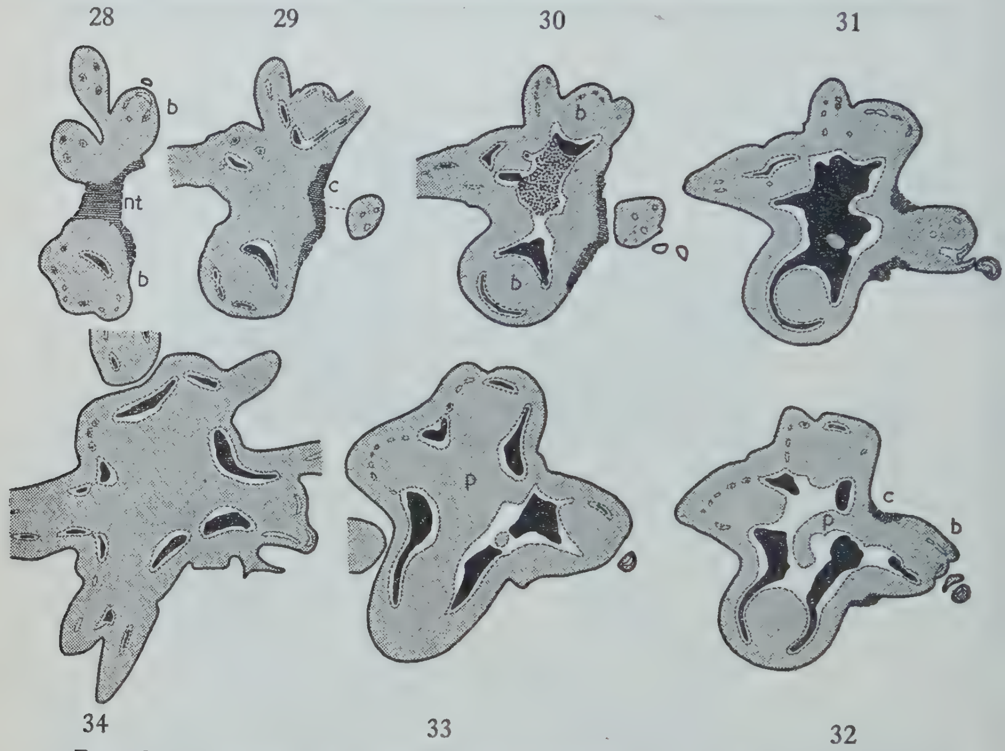


FIGS. 26, 27. Effects of treatment with 3-indoleacetonitrile (× 10).

FIG. 26. Transverse section of an apex which had been isolated by four vertical incisions and treated with 1/1,000 IAN-lanoline. The isolated apex became necrotic (*nt*) soon after treatment and large buds (*b*) developed in the subjacent region. A little below this level, Fig. 27, the vascular tissue of the lateral buds was seen to have spread right across the pith of the parent shoot. Xylem, black; phloem white; endodermis, broken line. Some of the necrosed and corky tissue (*c*) which formed round the incisions can be seen in this section.

These observations afford further evidence that IAN does not inhibit bud formation at the concentrations used: on the contrary, it may promote their growth and vascular development.

To summarize: 3-indoleacetonitrile greatly promotes the growth of the tissues and organs of the subapical region; the nascent and newly formed primordia round the base of the apical meristem may also be affected and may become extended and doubled in the tangential plane; directly or indirectly, treatment with IAN may lead to an attenuation of the apical meristem, but the latter nevertheless continues to produce leaf primordia in normal phyllotactic positions and sequence over a considerable period of time; bud formation and development are not prevented by IAN: on the contrary, buds may show vigorous growth and conspicuous vascular development.



Figs. 28–34. Effects of treatment with 3-indoleacetonitrile (× 10). Basipetal series of transverse sections of an apex treated as in Fig. 26, showing the curious configuration of the vascular tissues induced by the lateral buds in the pith of the parent shoot. *nt*, necrosed tissue of isolated apical meristem; *b*, bud; *c*, corky tissue at incisions; differentiating xylem, heavy stippling (Fig. 30); xylem, black. Below the level of Fig. 31 the pith vascular tissue becomes medullated (*p*) and the normal cross-sectional pattern is gradually regained.

(*c*) *Effects of 3-indoleacetic acid* (*IAA*)

The treatments were parallel to those in which 3-indoleacetonitrile was used. No developments of special morphogenetic interest were observed in apices treated with 10 mg./l. or 1 mg./l. IAA.

At 1,000 mg./l., applied during 14 days, a dark discoloration soon appeared in all wounded areas, but both the intact and isolated apices remained viable. The intact apices gave rise to some double leaf primordia, and there was a very marked stimulation of growth in the subapical region and in the primordia subtended there. Greatly distended, blister-like cells were a conspicuous feature of the superficial tissue of the subapical region. In the course of 28 days the apical meristem had become flat and broad and after 35 days it had become so distended basally that P_1–P_5 appeared relatively small and widely spaced. The apical meristem had now become residual, I_1 being the only new primordium. In the isolated apices giant cells were conspicuous round the base of the meristem and there was evidence of bud development near the apical cell group. Unfortunately, these apices were lost during the next fortnight from eelworm and fungal attack.

Apices, both intact and isolated, treated with 100 mg./l. aqueous solution for a period of 21 days underwent a very great enlargement of the subapical region, with the result that the apical cone occupied the bottom of a cup-like depression, and there was very conspicuous root development (Pl. IV, Fig. 6).

In treated isolated apices, in which the incisions were made at the level of I_1–I_2, the existing uninjured primordia, P_1–P_6, &c., showed very great enlargement, especially tangentially, in the course of 21 days' treatment. The basal stubs of injured primordia also became greatly enlarged. Bud formation was not yet apparent, but root development was abundant. Meanwhile the incisions had enlarged into wide 'channels', with a corky superficial tissue, and the apical meristem was left in an isolated, sunken, central position; it showed no formation of primordia (I_3 being the first that could normally appear) and little scaly development. After 29 days from the beginning of the experiment, no primordium had yet appeared on the isolated apex, but buds were seen to be developing in the subapical region. In an untreated apex I_1–I_5 were observed after 27 days and I_1–I_6 after 32 days. In the treated apices, after 49 days, the subapical region was of large size and showed conspicuous bud and root development. The isolated apices were now flattish and scaly and had given rise to primordia I_3–I_6 in different instances (Text-fig. 35). These were small but occupied approximately normal phyllotactic positions.

In 1954 an aqueous solution of IAA (100 mg./l.) was applied to isolated apices in the same manner. Six apices from one batch, on being fixed and sectioned, yielded results very similar to those obtained with IAN. The growth of the isolated apices may have been retarded but it was not suppressed; lateral buds were not inhibited; pith parenchyma was enlarged, especially in some leaf bases, and the vascular development tended to be more conspicuous, or, at least, to stain more conspicuously.

In 1952, 1/1,000 indoleacetic acid in lanoline was applied to apices in various ways:

IAA applied below the I_1 and I_2 positions. In this experiment, following the earlier work of M. and R. Snow and of Ball, small blobs of IAA-lanoline were placed in definite positions on the shoot apex: in the present experiments they were placed just below the I_1 and I_2 sites. Definite responses were obtained: I_1 (and I_6 in its vicinity) and I_2 became conspicuously enlarged so that the apex was tilted away from the side of the axis occupied by them (Text-figs. 36–40). Also leaf-gaps were widened, the pith cells of the leaf-base were enlarged, and the meristeles of the shoot seemed to be of smaller relative size.

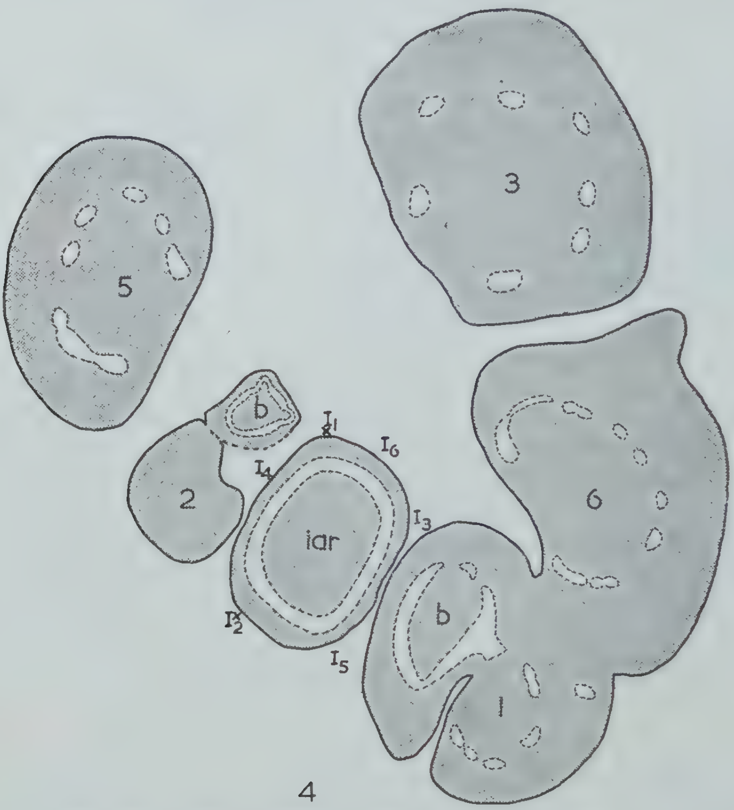
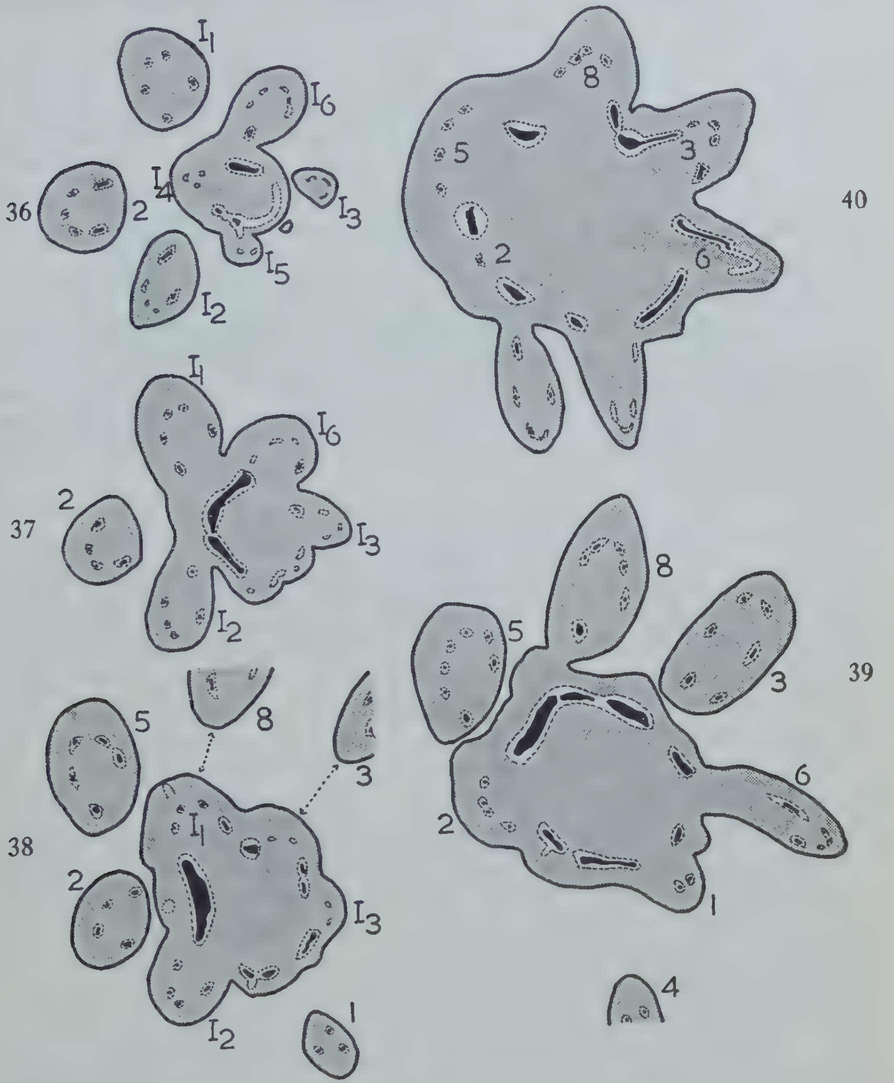


FIG. 35. Cross-section of apex showing the effect of treatment with 100 mg./l. 3-indoleacetic acid, the apical meristem having been isolated by four vertical incisions at the level of I_1–I_2. The primordia present at the beginning of the experiment (1–6) have become very large and widely separated from the isolated apex, and some buds (*b*) have been formed. On the isolated apical meristem, no primordia were formed at I_1 or I_2, but in sections above the level of that illustrated here, I_3–I_6 could be seen in normal positions. Incipient vascular tissue: white (×8).

In those apices in which the apical cell was damaged, strongly growing buds were formed with associated vascular tissue extending into the pith of the parent shoot. The records show that where the apex was damaged the application of IAA did not prevent bud formation but apparently enhanced the vigour of bud growth.

IAA applied to apices isolated by four vertical incisions. In some of these experiments the apical plugs were isolated so as to include P_1–P_5 and smeared with IAA-lanoline. These apices usually grew on and developed into short leaf-bearing shoots. The latter, as seen in transverse section, were characterized by the presence of wide leaf-gaps and by a reduction in the relative size of the shoot meristeles (Text-fig. 48). Buds were present in the subapical region, i.e. below the incisions. In those leaf primordia which were present on the plug at the beginning of the experiment, i.e. P_1–P_5, conspicuously enlarged medullary cells were typically present. In this experiment, as in those in which IAA was applied in aqueous solution, there was no evidence



FIGS. 36–40. Effects of 1/1,000 3-indoleacetic acid in lanoline applied as small blobs just below the I_1 and I_2 positions. The serial sections show that these two primordia (and also I_6) had become abnormally large (× 10).

that this substance directly affects the upper part of the apical meristem nor that it changes the morphogenetic pattern in any fundamental way.

In apices in which the incisions were made about the level of I_2, the isolated meristem, after treatment with IAA-lanoline, typically became necrotic. Large buds developed close to the incisions in the subapical region and subsequently, when transverse sections were prepared, various patterns of medullary vascular tissue were observed (Pl. IV, Fig. 9). The apex illustrated in Text-figs. 41–46, in which a layer of pith parenchyma underlay the necrotic distal meristem tissue, affords a good example of the way in which the outline of the bud-induced vascular tissue conforms with the perimeter of the shoot. These developments were typical of the several specimens (see also Text-figs. 47, 48).

To summarize: while there is clear evidence that IAA may be taken up by the superficial tissues in the transition and subapical region, there is no definite evidence that it is taken up by the more distal tissue of the meristem; it brings about a rapid increase in the rate of growth of the subapical region and promotes a conspicuous development of roots; it does not arrest bud formation and development, but may even stimulate bud growth and vascular development.

(*d*) *Effects of dinitrophenol, maleic hydrazide and coumarin*

It was thought that these and other substances which are known to inactivate enzymes and to inhibit growth might yield results of morphogenetic interest when applied directly to the apical meristem of *Dryopteris*. In the matter of concentrations, the writer selected those which are known to have lethal effects on the more delicate apex of *Marsilea* (Allsopp, 1955).

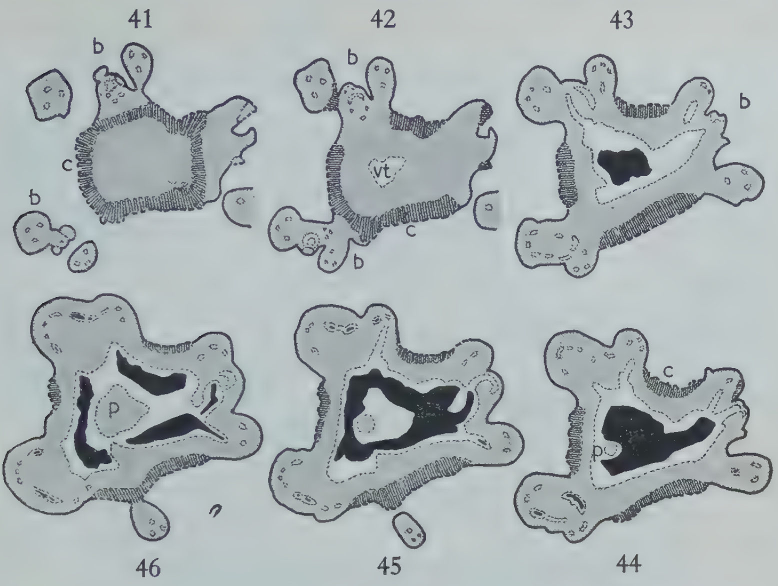
Dinitrophenol (*DNP*). As this substance yielded interesting results in the initial experiment, confirmatory evidence was sought, so that, in all, intact and isolated apices from six different batches of material were investigated. 100 mg./l. was found to be a useful concentration. Apices were soon killed by 1,000 mg./l. solution, and showed slight responses only to 10 mg./l. solutions. From an early season apex, collected 15 May 1955, and treated for 22 days, the following record was obtained (Text-figs. 49–51; 55, 56). (All the times stated are from the beginning of the experiment.)

After 14 days: little growth or development of scales; I_1 just appearing.

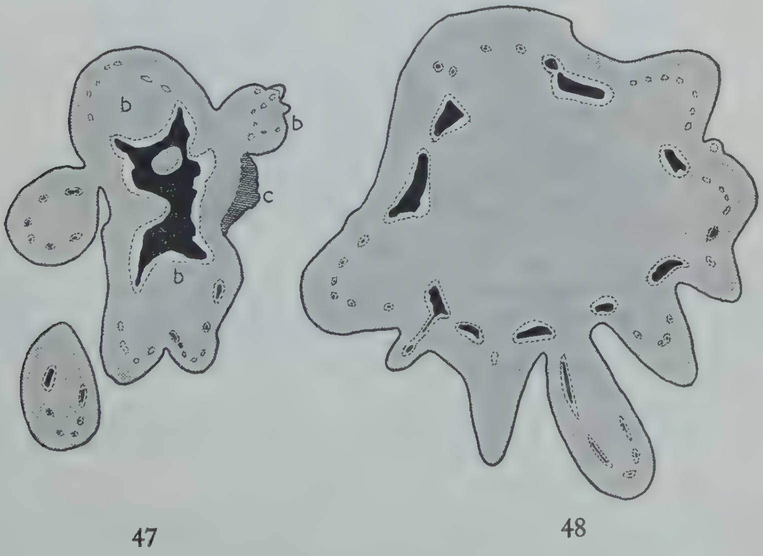
After 20 days: P_1 has broadened out and has a mound-like appearance as if developing into a bud; I_1 has now appeared; little development of scales.

After 36 days: P_1 is now quite bud-like, but has not yet formed leaf primordia; I_1 has widened tangentially as if forming a double apex; I_2 has appeared and is flat and bud-like; the older primordia, P_2, P_5, P_6, have widened tangentially as if forming double leaves (Text-fig. 49).

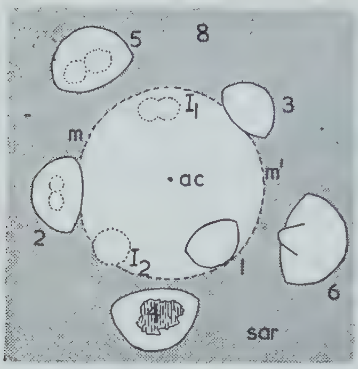
After 44 days: P_1 is now demonstrably a bud, its second primordium probably forming a double leaf; I_2 of the main apex appears to have a double apex, due to the development of a large distal scale; I_1 shows a similar formation (Text-fig. 50).



FIGS. 41–46. Transverse sections in basipetal sequence illustrating the effects of 1/1,000 3-indoleacetic acid in lanoline on an isolated apex. The isolated apex became necrotic during the treatment, buds (*b*) were formed in the subjacent region and gave rise to vascular tissue in the pith of the parent shoot. Lower down, this medullary vascular tissue itself became medullated and conjoined with the normal dictyostelic vascular system of the shoot. *c*, corky tissue marking the position of the four incisions; *vt*, bud vascular tissue in pith of parent shoot; *p*, pith; xylem black; endodermis, broken line (×8).



FIGS. 47–48. Treatment as in Figs. 41–46, illustrating a curious vascular development induced in the pith of the parent shoot (×8).



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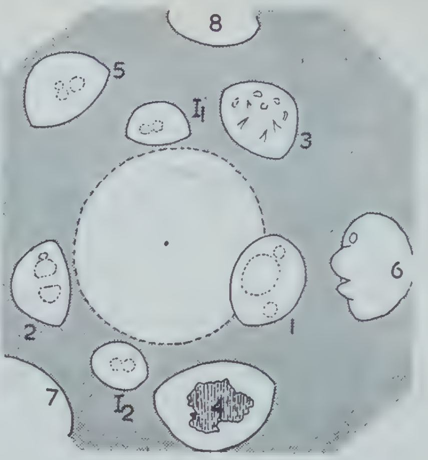

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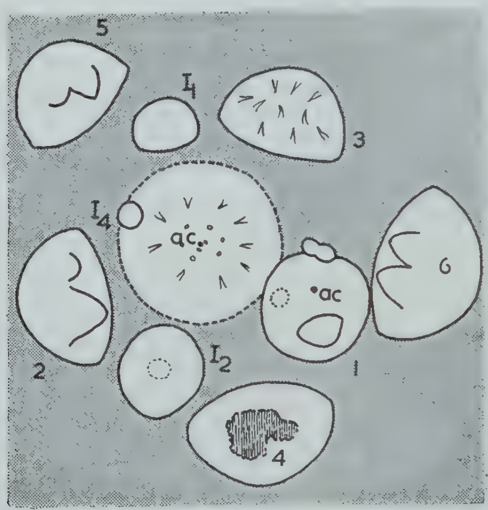

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FIGS. 49–51. Apex as seen from above after treatment with dinitrophenol (× 20).

FIG. 49. Several primordia, including the newly formed one at I_1, are showing evidence of double apices; I_2 has the appearance of a radial or centric leaf; P_4 has been damaged. *ac*, apical cell; the approximate outline of the base of the apical cone is indicated by the circular broken line (mm^1); *sar*, subapical region.

FIG. 50. The same apex, later. Leaf primordium (P_1) is developing into a bud, on which two nascent leaf primordia can be distinguished; P_3 has become scaly all over; I_2 appears to have a double apex.

FIG. 51. The same apex, still later. The transformation of P_1 into a bud is now fully confirmed, one of its leaf primordia being double; I_2 now appears as an awl-like radial leaf; the shoot apical meristem is becoming scaly right up to the apical cell; no primordium has appeared at I_3, but one has been formed at I_4.

After 50 days: the P_1 bud is now large and conspicuous and has three primordia; I_2 of the main apex has a sharply conical appearance and resembles a radial leaf; a scaly development has now taken place over a considerable part of the primary apical meristem and the beginning of scale formation can be detected near the still intact apical cell (Text-fig. 51).

After 64 days: the P_1 bud has now 5 primordia; I_2 is now slightly inturned and is more leaf-like in character; the main shoot apex is now scaly right up to its apical cell. The apex was fixed at this stage.

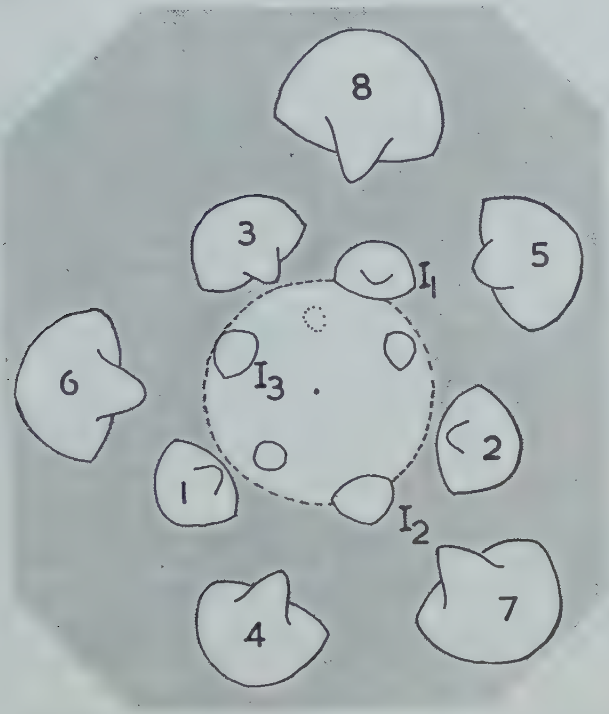
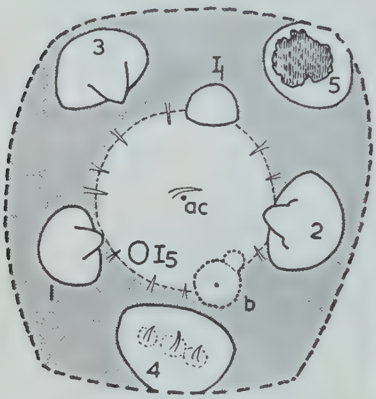


FIG. 52. A control apex from the same batch as Figs. 49–51 for comparison (see text).

Three out of four apices of the batches collected in May showed comparable developments, including bud induction in apices with a scaly but otherwise intact apical cell. In one apex which had been isolated and in which P_1–P_5 were present on the apical plug, there was little evidence of growth during the first 36 days, but a leaf primordium was formed at I_1 and scales developed near the tips of P_1, P_2, and P_4. By the fiftieth day a considerable enlargement of the plug had taken place, a scale had grown out in the vicinity of the apical cell, and a bud with one primordium was present in the I_2 site (Text-figs. 53, 57). There was some irregularity in the sequence in which the primordia of the main apex appeared, but after 64 days, I_3, I_4, and I_5 were observed in their normal positions, I_5 being larger than I_3 and I_4. A large, broad scale was now present at the tip of the shoot and another bud had appeared close to it. A bud which had been formed in the subapical region outside the incisions showed a similar scaly development and loss of apical growth potential. Thus the effects induced in an isolated apex by DNP were closely comparable with those observed in a treated intact apex. The DNP may have entered the cells of the apical meristem directly, or it may have gained access by way of the incisions, or by way of the parenchymatous tissue abaxial to P_3, P_4, P_5.

When these materials were duly fixed and sectioned, the observations recorded above were confirmed. Text-figs. 55–57 illustrate the large buds formed in two of the specimens. It will be seen that I_2, which appeared to be a radial or centric leaf under the binocular microscope, had a horseshoe-shaped leaf-trace but did not have the tangentially extended leaf-base typical of the normal primordium in *Dryopteris* (Text-fig. 55).



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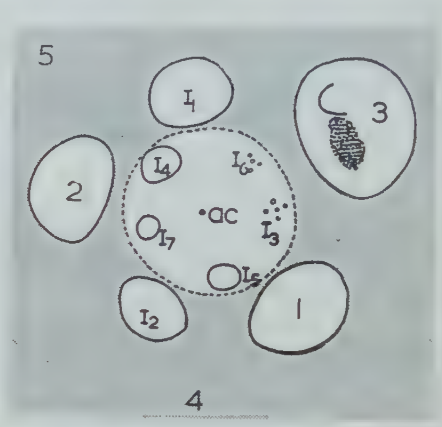

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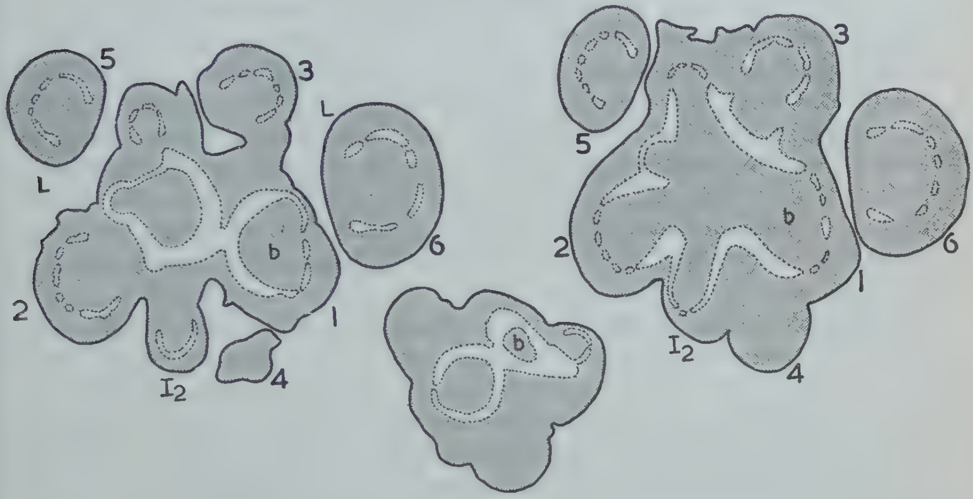
FIG. 53. An isolated apex, after treatment with dinitrophenol, as seen from above. A large scale has developed in the vicinity of the apical cell (*ac*) and a bud (*b*), with one leaf primordium, can be seen in the I_2 site. I_3 and I_4 have not yet appeared, but I_5 can be distinguished (×20).

FIG. 54. An apex, after treatment with dinitrophenol, as seen from above. The formation of leaf primordia at I_3 and I_6 has been suppressed, scales having developed over these sites (×20).

Treated intact apices from a batch collected in early June showed no special features after 14 days, but after 21 days' treatment there was some scaly development, I_1 had appeared, and there was evidence of growth in the subapical region and of the tangential enlargement of primordia. After 50 days one apex had become very flat but it did not become scaly to the tip. In a second apex, I_1–I_5 had been formed and there were no evident abnormalities. It should be noted, however, that the treatment of these apices had been somewhat intermittent. Further treatment was given for some time after the forty-second day of the experiment. By the fiftieth day there was some advance of the scaly development towards the tip in one apex, but a second apex remained apparently unaffected. These apices were given further intermittent treatments, e.g. on the seventy-fifth day of the experiment, by which time

I_1–I_8 had appeared, but the apices continued to grow, apparently normally, and this continued until the experiment was eventually concluded after 110 days. Such observations, when compared with those recorded for apices collected and treated earlier in the season, show that the state of the apical meristem, especially in the matter of its growth vigour, and continuity of application of the DNP, may both be important in determining modifications in the normal morphogenetic process.

At the beginning of July apices in which the apical meristem was still covered in by about 20 young leaf primordia were treated with three different concentrations of DNP, viz. 1,000, 100, and 10 mg./l. After 9 days of continuous treatment, all the apices were laid bare. Those which had been subjected to the highest concentration were found to have been killed, but no special effects were observed in the others. In one apex in which treatment (100 mg./l.) was continued for 45 days, growth was slow and there was a scaly development at the apices of I_1, I_2, and I_4. No primordium was formed in the I_3 site, the latter being occupied by scales. Later, in the same sector of the meristem, the I_6 site became scaly, though I_7 in due course duly yielded a primordium (Text-fig. 54). Thereafter the apex appeared to grow normally. Seventy-six days from the beginning of the experiment it was still growing normally and forming new primordia. During this period the control apices typically showed rapid growth and primordium formation, a new primordium being formed each 5–6 days.



55 57 56

FIGS. 55–57. Transverse sections of apices on which buds had been induced by treatment with dinitrophenol.

FIGS. 55, 56. Illustrate the disposition of tissues at two levels in the apex shown in Fig. 51: the vascular system of the large bud (*b*) which developed from P_1 is clearly seen; *l* normal leaf-base. Note the restricted development and somewhat anomalous vascular system of the centric leaf I_2.

FIG. 57. Illustrates a cross-section of the isolated apex shown in Fig. 53 (× 10).

Apices from a further batch, collected towards the end of July, were laid bare and treated as follows: *A*, left intact, *B*, isolated by widely separated incisions so as to include P_1–P_5, *C*, isolated by close incisions, so as to exclude P_1–P_5, and *D*, left intact. The *A*, *B*, and *C* apices were treated with 100 mg./l. DNP as already described; the *D* apices were not treated, but the base of the rhizome was freshly cut across so as to leave a rhizome piece 3 cm. long; this was placed in a dish of the solution 1 cm. deep. At intervals of 4 days the rhizome surface was freshly cut and immersed.

After 22 days the apices of the *D* materials, i.e. standing in DNP, had formed some four new primordia, but all the developments observed were normal and closely comparable with those in the control apices. The *A*, *B*, and *C* apices were all damaged and eventually killed by the DNP. (It is thought that these apices may have been somewhat desiccated at the time of treatment and that in consequence there may have been an excessive uptake of the DNP.) The *D* apices and some other untreated apices from this batch were given apical applications of DNP for 15 days during the latter half of August. After 35 days I_1–I_8 had been formed and the apices appeared to be growing normally.

Apices collected on 29 July 1955 were incised as in *B* and *C* above, but the DNP solution (100 mg./l.) was not applied to them until after 6 days, when the incision wounds and those incurred in preparation had become covered with a corky layer. Treatment was continued for 25 days, at which time there were various indications that the DNP was having an effect. As compared with the similarly incised control apices, there was a considerable slowing down in the rate of growth, e.g. treated apices had only formed I_1 and sometimes I_2, whereas I_1–I_6 had been formed in the controls. Also, some of the newly formed primordia in the treated apices were extended tangentially and had a scaly development at their apices. However, the morphogenetic activity of the treated apices did not remain depressed and during the following 22 days primordia up to I_4 were formed in the *B* and *C* apices. During a further 15 days, primordia up to I_8 were formed in *B* apices and up to I_6 in *C* apices. In short, after the initial phase, the DNP did not appear to have had much effect.

These observations on DNP-treated apices from different batches have been set out in some detail because they afford some idea of the variable reaction of apices subjected to the same treatment at different seasons.

To summarize: according to the reactivity of the apex and, presumably, in relation to the amount of DNP taken up by the tissues in or near the apical meristem, a more or less considerable retardation of growth of the apical meristem may result; buds may be formed instead of new leaf primordia and even instead of P_1; scales may develop on the apices of young primordia, in primordium sites (with suppression of primordium formation) and at the tip of the shoot; in actively growing apices, or in apices in which only a small amount of DNP has been taken up, these abnormal developments may be absent, slight or transitory.

Maleic hydrazide. Maleic hydrazide was applied in aqueous solution

(100 mg./l.) to intact and isolated apices from four batches collected in May, June, and towards the end of July, the treatment being continued up to one month. The concentration used is known to be lethal to the delicate apices of *Marsilea* (Allsopp, 1955).

In apices collected and treated during the first half of May, growth was very slow; I_1 only appeared after 15–18 days, and there was little growth or development of scales at the base of the meristem. By the twenty-fourth day a pimply or blister-like development was observed round and on the youngest primordia, and this gradually spread upwards towards the tip of the apical meristem. Some growth was now apparent in the subapical region, and tangentially extended and double primordia began to be formed. In the course of 47 days the pimply development had become very conspicuous in some apices, so much so that primordia P_1 and P_2 had become obscured or virtually obliterated by it. Although an occasional new double primordium was formed, there was very little growth in these apices. The developments recorded above were typical of both the intact and isolated apices. When these apices were eventually fixed after 87 days they still showed little evidence of growth, very few scales had been formed, and the pimply development was present in the region of the apical cell. On being sectioned they proved to be very crusty and almost moribund. The pimples were seen to consist of greatly distended, superficial cells.

In apices collected and treated in June some double leaf primordia developed in the course of 42 days and scales were formed on the apical meristem, including the region of the apical cell. These specimens showed considerably more growth activity than did the May apices. The pimply development noted in the May apices was less conspicuous or absent in some apices. After 61 days one apex had given rise to a bud in the I_3 site, and after 75 days another apex yielded the record illustrated in Text-fig. 58: eight new primordia had been formed; a scale was present at the apical cell and a bud had been formed above the axil of I_6; the site of I_5 was occupied by two primordia or lobes, and I_2 and I_7 had the appearance of double and treble primordia respectively and were extended in virtually a radial direction from the apical cell.

A further group of apices, collected and prepared on 27 July, was treated as before, meristems being incised so as to include or exclude P_1–P_5 in different instances. Treatment with maleic hydrazide was continued for 30 days. In the course of 14 days it was already apparent that the untreated control apices, in which primordia had appeared at I_1 and I_2, were growing more rapidly than the treated ones. After 29 days both the intact and isolated treated apices, which, by comparison with the controls, afforded further evidence of growth arrest, were still without any considerable scaly development and were becoming pimply. Some double primordia were present in the intact apices on which I_1–I_4 had now been formed. After 35 days (when eight new primordia had been formed in the intact control apices and five to six in the isolated control apices) primordia I_1–I_5 had been formed in the treated apices.

These showed the characteristic pimply development, i.e. maleic hydrazide does not prevent the inception of leaf primordia but it may arrest and modify their further development. In the treated isolated apices the pimply development was found both in the isolated apical meristem and on the meristems of the buds that were developing in the subapical region. In one of the apices isolated by close incisions, i.e. excluding P_1–P_5, a scaly development was observed at the apical cell. This batch of apices, though showing greater activity, thus yielded substantial confirmation of the observations made earlier in the season.

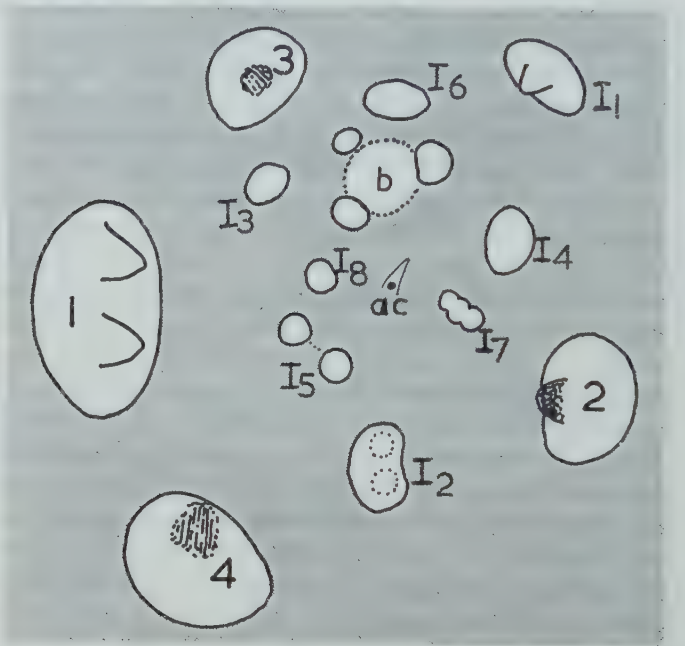


FIG. 58. An apex, after treatment with maleic hydrazide, as seen from above. The apical meristem became scaly. A scale is present at the apical cell and a bud has been formed above the axil of I_6. Some double leaf primordia can also be seen (see text) (×20).

To summarize: maleic hydrazide may cause a more or less considerable arrest of growth in the apical meristem; primordia may continue to be formed but their further development may be modified, and they may even be obliterated, by the development of pimply or blister-like superficial outgrowths; in some apices a scaly development of the meristem took place, including the apical cell, and some bud formation was induced; the tissues of the more severely affected apices was reduced to a crusty, moribund condition.

Coumarin. The effects of coumarin (100 mg./l.) have only been investigated on small numbers of apices collected in May. Treatment was continued for 25 days. By that time primordia had been formed at I_1 and I_2 and the normal development of scales had taken place. After 41 days P_4 and P_5, P_6, &c., were seen to be of large size relative to the inner primordia, and the apices of some of the youngest primordia showed evidence of a scaly development. After 48 days new primordia I_1–I_5 had appeared and were abnormally small relative to P_1–P_5. At this time one of the isolated apices had become scaly to the summit and looked as if it might form a bud; lateral buds were present in the subapical region. After 80 days the apices were scaly well up to the tip but no buds had been formed on the meristem. At the concentration used, coumarin thus appears to have effects generally like those of dinitrophenol.

Sodium arsenite. Some preliminary observations indicate that this substance may yield observations along the same general lines as those recorded above.

(*e*) *Effects of yeast extract and of thiamine hydrochloride*

Yeast extract. In most of these experiments a concentration of 500 mg./l. (unautoclaved) was used, this having been reported as yielding marked increases in the rate of growth of excised fern apices in aseptic agar media (Wetmore, 1953, 1954). Apices from five batches, collected between May and the end of July, were treated for periods up to 21 days.

During the first 21 days treated intact apices from the May and early June collections broadened out, this effect being still more evident after 34 days. As a result the younger primordia, P_1–P_7, became widely separated and appeared to be of small size relative to P_8–P_{13}. At this stage the development of scales was still normal, i.e. it was confined to the lower part of the apical cone. By the forty-second day of the experiment, however, the apical meristem, which was now very much flattened out, had become scaly almost to the apical cell. The leaf primordia P_1–P_3 and I_1–I_5 (new leaf primordia) had now become considerably enlarged and there was evidence of bud formation at several points on the meristem (Text-figs. 59, 60). Occasional centric leaves were also observed (Text-figs. 61, 63–65). After 62 days the apex was flat and scaly all over but bud growth remained relatively feeble. These several developments were confirmed when the materials were fixed and sectioned transversely and longitudinally (Pl. IV, Figs. 1, 2, 5). It was seen that the pith parenchyma had been in a state of active division at the time of fixation. In those specimens in which the distal region of the axis was still unchanged, i.e. had not yet become scaly, the apical cell, the prism-shaped superficial cells of the meristem, and the incipient vascular tissue appeared to be normal and active (Pl. IV, Fig. 1). But in other specimens the embryonic character of the distal region had been lost, the apical cell had become much divided and had given rise to a strongly growing scale, and the subjacent cells were dividing and enlarging into parenchymatous tissue. Yeast extract applied in this way appears to stimulate a precocious development of parenchyma and scales in the meristem (Pl. IV, Figs. 2, 5). These histological developments will be considered in greater detail in a later paper.

In the treated isolated apices of the same batch this scaly development of the meristem was considerably less marked. In some of them tangentially extended double leaves had been formed and double leaves were also observed in the buds which had developed in the subapical region outside the incisions (Text-figs. 67–69).

In materials collected early in July yeast extract was applied at concentrations of 1,000, 500, and 250 mg./l. to intact apices. The observations recorded in the ensuing 6 weeks were in substantial agreement with those noted above: in specimens where the shoot apical cell had given rise to scales buds were formed (Text-figs. 63–66). The reactions of apices treated with the two lower concentrations were closely comparable. At 1,000 mg./l. apical

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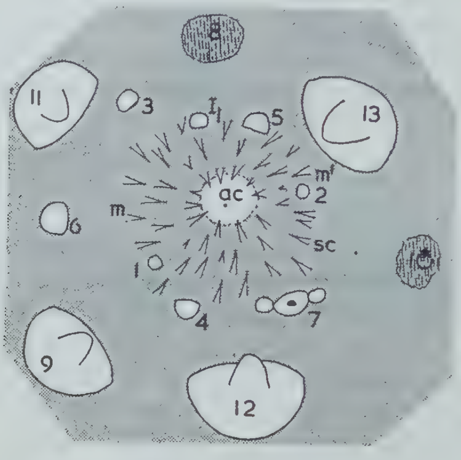

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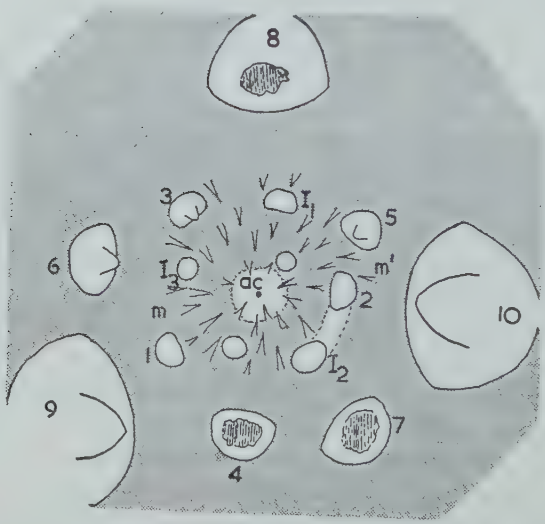
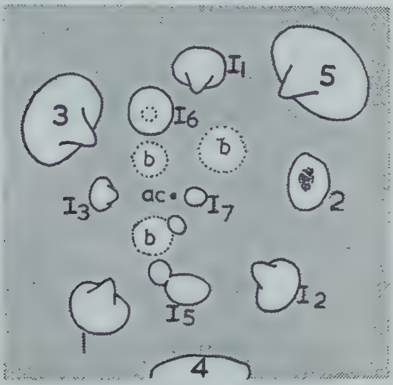


Figs. 59, 60. Apices, after treatment with yeast extract, as seen from above (× 13).

The original conical apex, indicated approximately by mm^1, has become rather flattened and scaly (*sc*) close to the apical cell (*ac*); primordia P_1–P_7 and the new primordium at I_1 have remained small and have become relatively widely separated. In Fig. 59 the apex of P_7 was slightly injured and two lateral primordia have formed; P_8 and P_{10} were damaged at the outset.

growth was slower, and after a time the apical cell in several specimens became faintly outlined in brown—an indication of the onset of necrosis. In apices collected towards the end of July the tissues surrounding the isolated apical plug in some specimens were removed and, to admit of the development of wound tissue, 6 days were allowed to elapse before the yeast solution (500 mg./l.) was applied. Both the isolated and intact apices began to exhibit the characteristic abnormal leaf developments from the twelfth to the twentieth day after the beginning of the treatment, the scaly development of primordia being a conspicuous feature. Some of the apices, including some of those isolated above P_1–P_5, became scaly to the extreme tip, but others gave rise to leaf primordia in sites I_1–I_6 and still had a small apical meristem free of scales when the experiment was concluded (Text-figs. 59–62).



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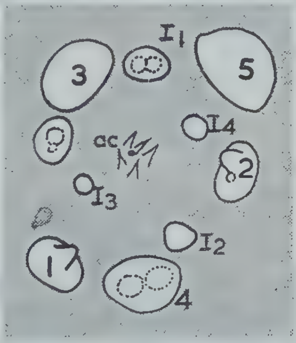

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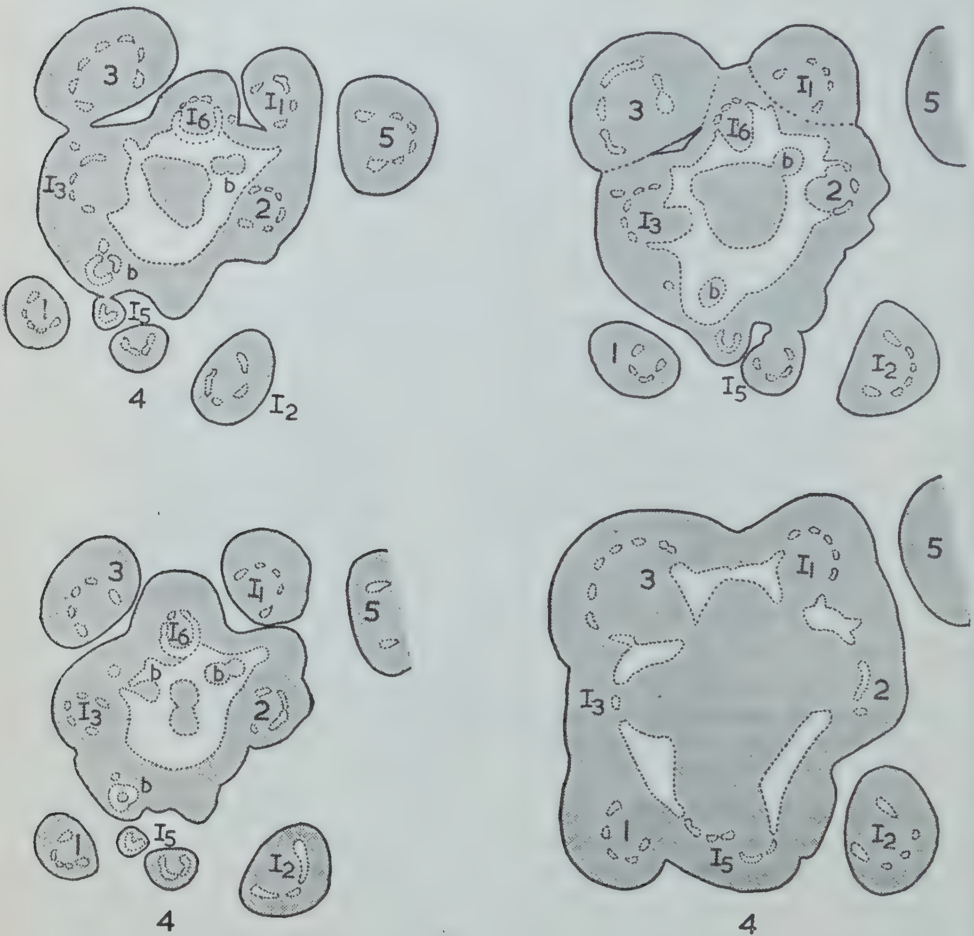
FIG. 61. The whole of this apical meristem, including the apical cell group (*ac*), became scaly. New leaf primordia, up to I_7, were formed and three buds (*b*) could be distinguished.

FIG. 62. The whole of the apical meristem, including the apical cell group (*ac*), has become scaly; some double primordia can be seen.

Thiamine hydrochloride. In this survey it was not feasible to test the effects of the several ingredients of yeast extract and, accordingly, thiamine hydrochloride was selected as a substance that might prove interesting. When applied in aqueous solution at 100 mg./l., it yielded results which were generally comparable with those obtained with yeast extract. Some young leaf primordia became double and scaly in their apical region and the shoot apical meristem eventually became scaly to the tip (Text-figs. 70, 71). After 36 days, in one of the isolated apices, the I_1 site, which was on the subapical side of the incision, had developed into a bud; P_5–P_9 no longer had active apices and had become scaly mounds; P_7 had the appearance of a scaly centric leaf; and P_6 had become 3-lobed, due to a precocious development of pinnae. On the isolated meristem, however, leaf primordia had been formed in the normal I_2–I_5 positions. After 56 days this apex still appeared to have been little affected by the treatment, whereas the intact apex had become scaly right to the summit.

Intact and isolated apices, from a batch collected at the end of July, were treated in early August, a period of 5 days being allowed for wound healing. The observations made were in general agreement with those recorded earlier in the year. After 50 days both the intact and isolated apices were typically scaly up to the apical cell. Some of the results obtained with thiamine hydrochloride are illustrated in Pl. IV, Figs. 3 and 4.

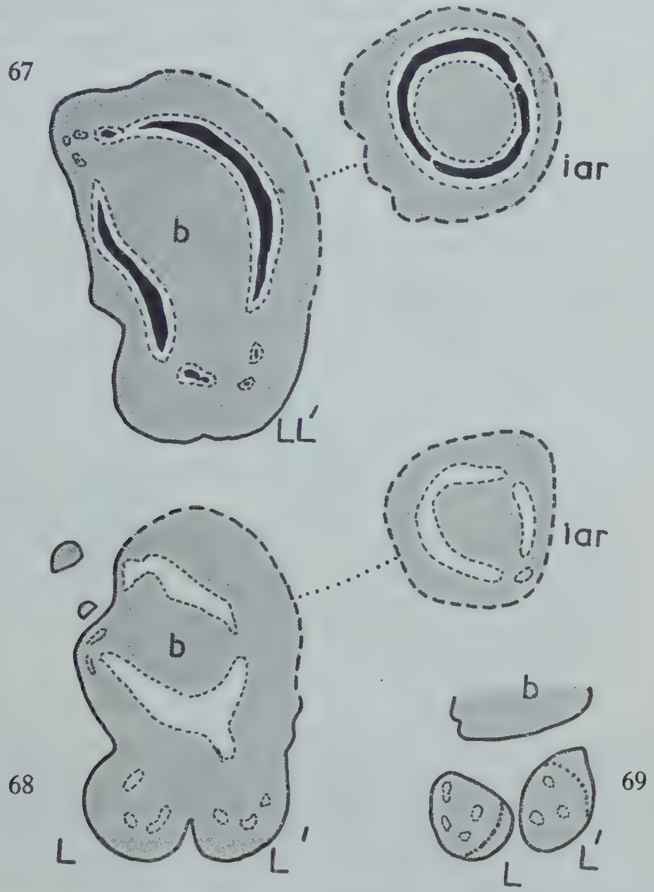


FIGS. 63–66. Effects of treatment with yeast. Transverse sections in basipetal sequence of the apex illustrated in Fig. 61. The three buds (b), the centric leaf which formed at I_6, and the vascular system of the double primordium which formed at I_5, can readily be distinguished. One of the buds occupies approximately the I_4 position; the other two are situated above the axils of P_1 and P_3 (× 10).

(*f*) *Effects of other substances*

Various other substances have also been applied to small groups of apices to obtain indications of their possible morphogenetic interest (see Table 1).

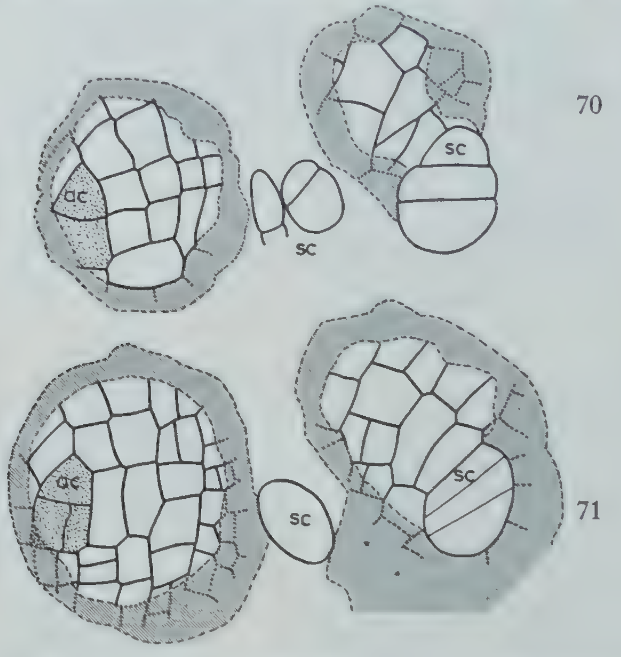
The indications are that the apical meristem will probably show considerable reactivity when treated directly with a varied range of organic substances. With adenosine triphosphate there was a scaly development at the apex of young leaf primordia and a precocious scaly development of the apical meristem. Although many apices were treated with solutions of tri-iodobenzoic acid and trichlorobenzoic acid during 1952, 1954, and 1955, no developments of special morphogenetic interest were induced. These substances yielded some interesting and unusual developments when applied to dicotyledonous plants (Gorter, 1951; Wardlaw, 1953).



Figs. 67–69. Transverse sections, in acropetal sequence, illustrating an isolated apex after treatment with yeast extract. This isolated apical region (*iar*) is seen to be solenostelic below, Fig. 67, and dictyostelic higher up, Fig. 68. The conspicuous bud (*b*) which formed in the subapical region has given rise to a large double leaf (*LL′*) (× 15).

Desoxypentose-nucleic acid, at 100 mg./l., was applied to intact and isolated apices collected at the end of May and the beginning of June. After 28 days the intact apices had become broad and saucer-like, with the apical meristem occupying the base of the depression; i.e. the subapical region reacted as if to a growth stimulus. The primordia, including the new ones at I_1–I_3, appeared relatively small and widely separated (Text-fig. 72). Observations were continued for 70 days when the apices were seen to be very wide and flat, with a small, residual apical meristem and small primordia.

Glutamine (500 mg./l.) was applied for 28 days to apices collected as above. During the initial 21–28 days there was little evidence of growth in the apical meristems but, to cite the record for one of the intact apices, P_1 had become carbuncular and scaly, P_2 had become double, and P_4 was conspicuously extended tangentially; no primordium had yet appeared at I_1, but there was the beginning of a scaly development in and round the I_1 and I_2 sites. The isolated apices similarly showed little growth; in one specimen the primordium at I_1 developed as a circular mound and P_1 became scaly. After 28 days several double leaf primordia were present in the intact apices but no special effects were noted in the isolated ones. In the course of 56 days, P_1, I_1, I_2, I_3 had all developed as double leaves in one of the intact apices (Text-fig. 73).

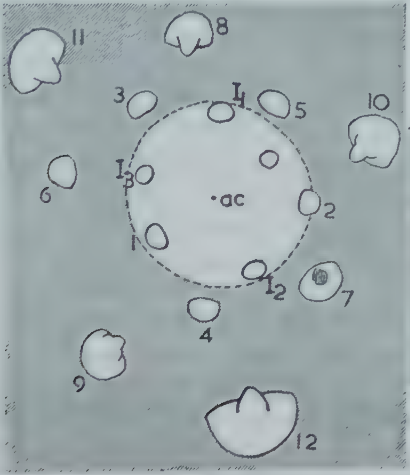


FIGS. 70, 71. Transverse sections of an apex which had been treated with thiamine hydrochloride. The sections, which are 10 μ apart, traverse the apical cell of the shoot (*ac*) and, as the apical cone had become much flattened, also the last-formed leaf primordium (on the right). Scales (*sc*), which covered most of the apical meristem, are already present on the adaxial and lateral sides of the primordium; the latter shows evidence of tangential extension and the characteristic, large lens-shaped apical cell cannot yet be distinguished (× 150).

V. DISCUSSION

The experiments described in the preceding sections show that the growth, organization, and normal morphogenetic activity of the fern shoot apex can be modified by the direct application to it of solutions of different organic substances. Some of the morphological developments recorded have not been reported by workers using the methods of organ- and tissue-culture.

Although, in the present experiments, the morphogenetic activity of the apical meristem has been modified in a number of ways, the writer would reserve judgement as to whether any of the substances thus far used has effected any fundamental change in the constitution or working of the apical reaction system—lethal effects apart. Even in apices in which a precocious parenchymatization and scaly development of the meristem were taking place, leaf primordia continued to be formed in normal phyllotactic positions for some considerable time. And again, when buds were formed on the meristem, they typically developed from P_1, or in an I_1 or other leaf or bud site. In short, buds appeared in what surgical experiments have shown to be possible bud positions in this fern species. Far-reaching changes in the morphogenetic activity of the apical meristem, comparable, for example, with the axial ring fasciations which Gorter (1951) was able to induce in the tomato apex by treating the plant with tri-iodobenzoic acid, have not so far been obtained in these investigations. It may be that the gene-determined reaction system of *Dryopteris* can only be modified within narrow limits, and in certain ways, and still remain functional.



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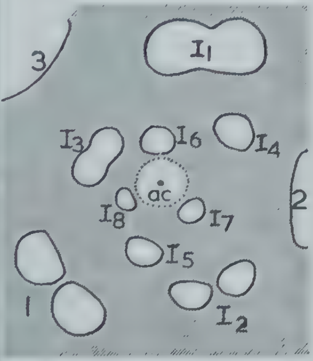

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FIG. 72. Apex, as seen from above, after treatment with desoxypentose-nucleic acid. The primordia have remained small and become widely separated on the saucer-like distal region (×20).

FIG. 73. Apex, as seen from above, after treatment with glutamine. The apical meristem has become scaly and reduced to small size. Double primordia P_1, I_1, I_2, and I_3 can be distinguished (×13).

The experimentally induced departures from the normal development can be referred to: (*a*) the arrest, or partial arrest, of growth in the apical meristem and, in particular, in the apical cell group; (*b*) the relative acceleration of growth in the subapical region; (*c*) increases and decreases in the activity of the growth centres which normally become leaves; and (*d*) the precocious parenchymatization of the apical meristem. In some instances more than one of these processes may be involved. Hence the arrest of activity in the apical cell group might be attributed either to the direct uptake by it of the physiologically active substance or to an acceleration of growth in the subapical region, with a consequential depletion of the metabolites normally utilized in the most distal tissues. Where bud formation has been induced, e.g. in apices treated with dinitrophenol or maleic hydrazide, an arrest in the normal regulative activity of the apical cell group may be inferred, these morphogenetic effects being closely comparable with those obtained by puncturing the apical cell. In apices treated with yeast extract, some bud induction was also observed and there was evidence that the embryonic character of the apical cell group had been lost and that a precocious development of parenchyma and scales had been induced. These histological developments are of special interest because they are typical of the differentiation that usually takes place in the region of transition from the apical meristem to the subapical region.

The formation of large, double leaf primordia, typically extended in the tangential direction, may be referred to an increase in the metabolic activity of the leaf growth centre. In some instances it appeared that the growth centre had enlarged and divided before the outgrowth of the primordium began. Not all double primordia, however, were of conspicuously large size. In some the doubling was due to the suppression of activity in the primordium apex and the precocious outgrowth of pinnae. In others, especially those found in diminishing and attenuating apical meristems with a rapidly widening subapical region, the tangential enlargement and doubling of primordia may have been partly due to the tangential tensile stress set up in the region between the slowly and rapidly growing apical and subapical regions respectively. Thus, in apices in which the rapid relative growth of the subapical region was sustained over several weeks, e.g. as a result of applications of IAN, several of the later-formed leaf primordia, e.g. I_{10}, were observed to be double. These double primordia had nevertheless been formed on a very small residual apical meristem. At this point it is also pertinent to note that wherever centric or radial leaf primordia were obtained, i.e. primordia in which the radial and tangential components of growth were approximately equal, the growth of the primordium was limited and it did not attain to any considerable size (see below).

A characteristic effect, due to a relative acceleration of growth in the subapical region, was obtained when substances such as 3-indoleacetonitrile and desoxypentose-nucleic acid were applied to the apex. This consisted in a vigorous upgrowth of the flattish subapical region, with the result that the apical cone came to occupy the centre of a more or less deep depression—the sunken apex (or *Scheitelgrube*) recognized by R. and C. Wetter (1954), the writer (Wardlaw, 1956), and others as being the normal configuration in the adult shoots of certain other ferns. In younger and smaller shoots of these ferns, however, the apex—connoting the distal region as a whole—is first conical and then flattish. The present investigation shows that, by direct chemical treatment of the apex, it is possible to induce morphological configurations that have a special interest both in their ontogenetic and genetical aspects. (Cf. Gavaudau *et al.*, 1951.)

With regard to the controversial topic of the control of bud inception and development by substances such as indoleacetic acid and indoleacetonitrile, the records show that neither of these substances, at the several concentrations used, inhibits bud formation, i.e. under experimental conditions in which buds would normally be formed. On the contrary, both substances may promote the growth of large, strongly vascularized buds.

If the normal, regulated development of the apical meristem is disturbed in characteristic ways by surgical means, e.g. if the normal disparity in the rates of growth on the adaxial and abaxial sides of a leaf primordium is removed, an organ of radial symmetry—a bud—tends to be formed (Wardlaw, 1949, 1955*a*; Wardlaw and Cutter, 1955; Cutter, 1954). If, however, the difference in these growth-rates were accentuated, e.g. by an acceleration of growth in the subapical region or by an arrest of growth in the apical meristem, a leaf primordium in the course of formation at the base of the apical cone would tend to undergo a more or less considerable tangential extension. In the case of a nascent primordium (i.e. a new primordium in very early plastochrone) the growth centre might become divided and two primordia would develop in a single leaf site; and a primordium in later plastochrone might be expected to give rise to a double or fasciated primordium. These and other related variants of normal leaf inception and development encountered in the present studies afford support for the writer's views on leaf symmetry and orientation. Only very occasionally have induced double primordia been extended in the radial direction.

The centric leaf primordia which have come under observation in these investigations have typically been small attenuating organs of limited growth. The marginal meristems disappear, the characteristic tangential widening of the primordium is arrested, and the apex also loses its embryonic character and is transformed into parenchyma. Concomitantly, the vascular system undergoes a number of changes: the crescentic group of meristeles as seen in a cross-section of the basal region becomes a dictyostelic ring which thereafter diminishes through a solenostelic to a protostelic state and eventually fades out distally. Such leaves are not truly radial: they are foliar members in which there has been a marked diminution of growth in the tangential plane. In that centric leaves may occur in apices in which the adjacent primordia have developed as large double leaves or as buds, partial inhibition and competition for nutrients may be among the factors which determine their characteristic development.

In *Dryopteris* the shoot apical meristem and the distal and marginal meristems of leaf primordia are usually quite free from scales. Scale formation is typical of those basal regions of the meristem where superficial meristematic cells are actively dividing and developing into cortical parenchyma. Scale formation thus affords a useful indicator of this phase of differentiation, and, accordingly, when the tip of a shoot or a leaf begins to show evidence of a scaly development it can rightly be inferred that its normal embryonic character has been lost. Unautoclaved yeast extract and thiamine hydrochloride, for example, may both induce a precocious parenchymatization and scaly development of the apical meristem, including the apical cell group. On the other hand, when autoclaved yeast extract is an ingredient of an agar tissue-culture medium it greatly promotes the growth of explanted fern apices, but it does not produce the effects mentioned above. When an explanted apex is growing in a solid culture medium the effect of a particular substance may be obscured or modified by the presence of other ingredients of the medium or by the need for it to pass through the basal callus and parenchymatous tissue of the explant.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATE IV

(All figures are from untouched photographs)

Dryopteris austriaca (*D. aristata*)

FIG. 1. Longitudinal section through the apical cell of an apex treated with yeast extract. A scaly and parenchymatous development has supervened upon most of the apical meristem, but a small residual meristem is still present at the summit of the shoot. The apical cell and some of its recent segments still persist and incipient vascular tissue can be distinguished immediately below them. Scales have developed very close to the apical cell (×225).

FIG. 2. Longitudinal median section of an apex which had been treated with yeast extract. The normal distinctive appearance of the apical meristem and of the apical cone have been lost, leaf primordia are very inconspicuous, and scales have developed right up to the summit of the shoot (×35).

FIG. 3. Longitudinal median section of an apex after treatment with thiamine hydrochloride. The apical cell, the prism-shaped cells of the superficial meristem, and the underlying cells have become much divided and scales have been formed all over the meristem including the region of the apical cell (×225).

FIG. 4. From the same apex as Fig. 3. This section, taken close to the apical cell, shows a well-developed scale high up on the apex and the prococious 'parenchymatization' of the whole distal region (×225).

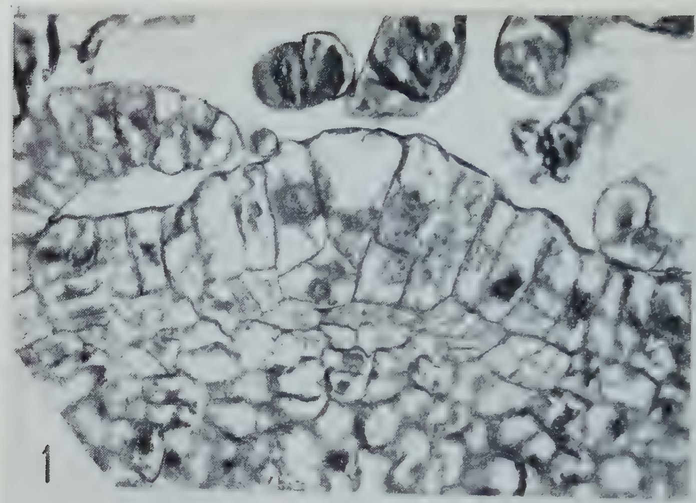
FIG. 5. The distal region of the apex shown in Fig. 2. The apical cell and the distinctive prism-shaped cells of the apical meristem can no longer be seen: a parenchymatous development has been induced, the apical cell has become much divided and has given rise to a large, deeply staining, spine-like scale (× 225).

FIG. 6. Longitudinal median section of apex after treatment with 3-indoleacetic acid. The small apical cone (*a*) is situated at the base of a cup-shaped depression—a result of the extensive growth-development induced in the subapical region (× 7).

FIG. 7. Transverse section of a shoot of which the apical meristem had been isolated high up and treated with 3-indoleacetonitrile in lanoline. The apex soon became necrotic and buds were formed in close proximity. The vascular tissue of these buds spread across the pith of the parent shoot as shown in this illustration (× 15).

FIG. 8. Treatment as in Fig. 7, showing an unbroken mass of bud-induced vascular tissue in the pith of the parent shoot.

FIG. 9. A cross-section illustrating the curious development of vascular tissue in the pith of the parent shoot when IAA was applied to an isolated apex which became necrotic soon after treatment. The vascular tissue pertains to three buds which developed close to the necrosed apical meristem. At the level of this section the medullary vascular tissue is itself becoming medullated; this increases basipetally till eventually the bud vascular tissue merges with that of the shoot (× 15).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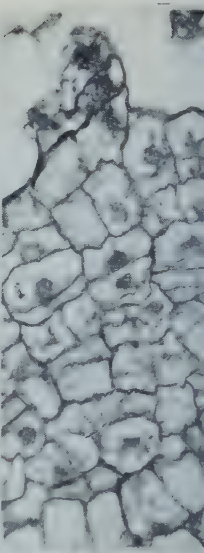

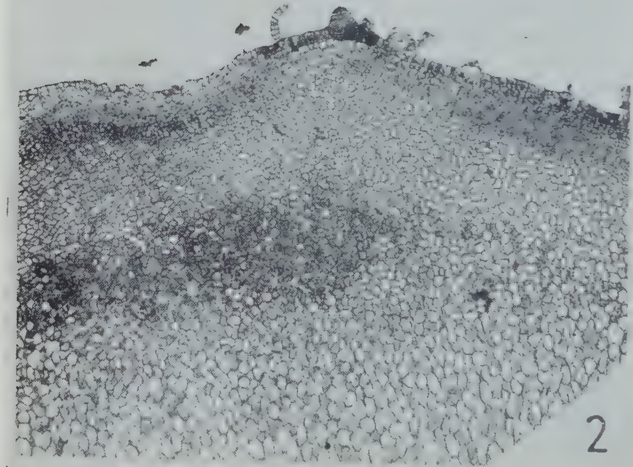
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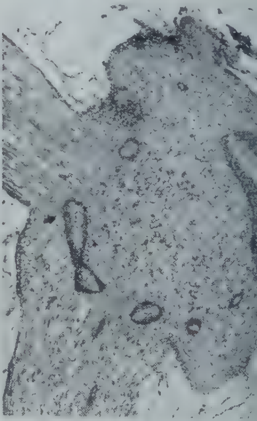

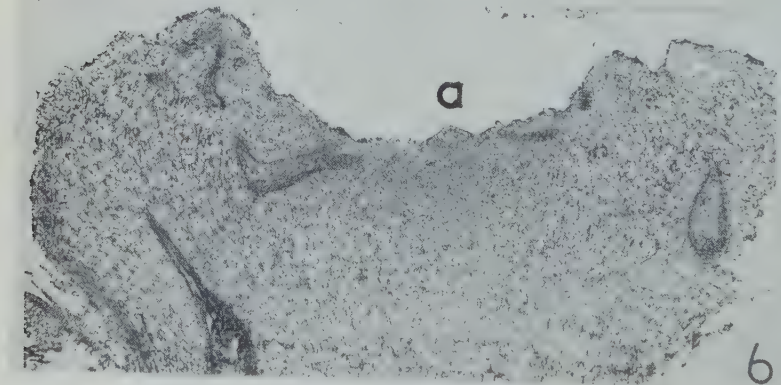

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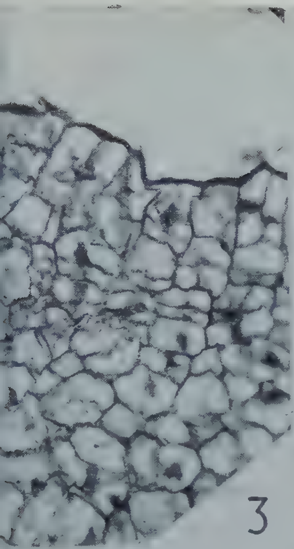

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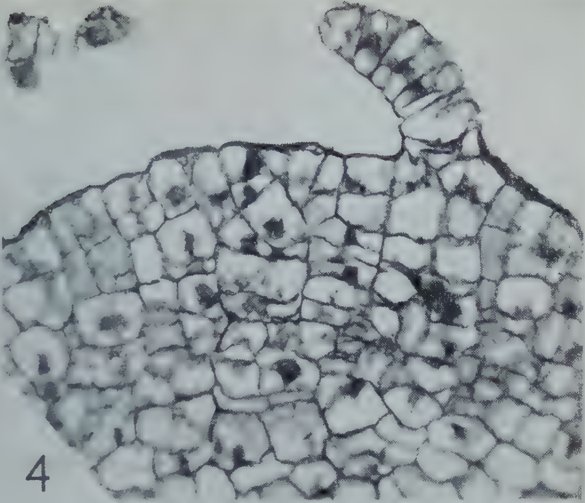

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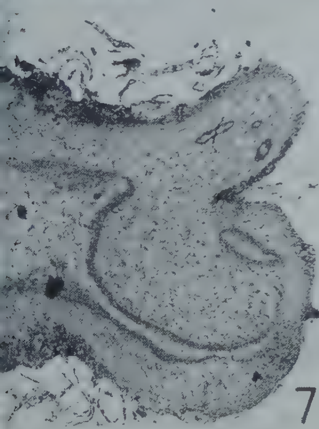

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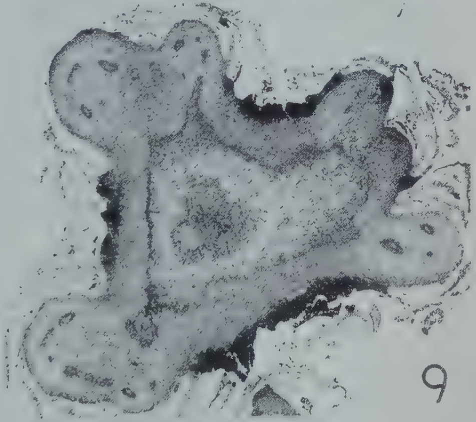

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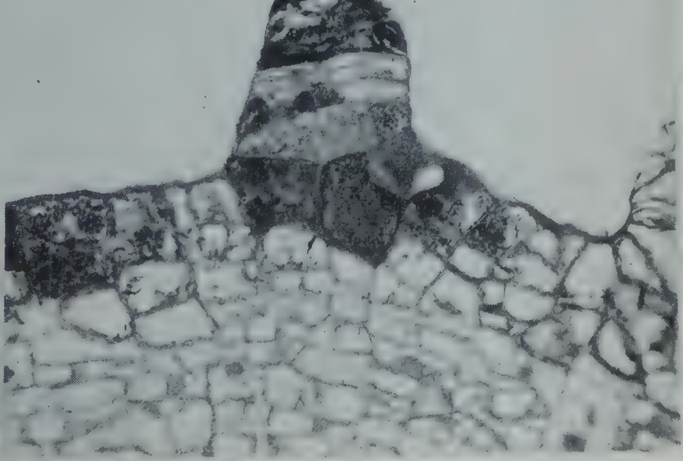

AW

The Effect of Carbon Dioxide on the Absorption of Manganese by Root Tissues of Red Beet

BY

A. D. SKELDING

(*Botany Dept., University of Birmingham*)

With ten Figures in the Text

ABSTRACT

The absorption of manganese by beetroot tissue is shown to be partially inhibited by the presence of carbon dioxide. Preliminary treatment of well-washed tissue slices with carbon dioxide also produces, after placing them in manganese solutions, an inhibition of absorption for a time similar to that shown by tissue slices when first excised from stored roots. It is suggested that an inhibitory substance is formed in bulky storage roots and tubers by dark fixation of accumulated respiratory carbon dixoide. On cutting thin slices from such organs, the removal of inhibitor may take place by diffusion into water during washing, by reversal of the fixation reaction when the partial pressure of carbon dioxide is reduced, and possibly also by oxidation to form a substance capable of stimulating manganese absorption when sufficiently high oxygen concentrations are available.

INTRODUCTION

THE observations presented here are a continuation of the investigations into the absorption of manganese by beetroot storage tissue reported by the author in an earlier paper. Skelding and Rees (1952) reported the extraction of a water-soluble substance from beetroot kept in store during the winter which prevented the absorption of manganese by thin slices of beetroot tissue. This substance, which has not yet been isolated, appeared to reach its greatest concentration in the roots at about the end of January. In roots taken freshly from the ground during October there was very little inhibiting principle present and by the end of the storage season in March when the roots were sprouting actively the substance had almost disappeared. This rise and fall in the inhibitor content of the roots suggested a possible connexion with seasonal dormancy which was further supported by the fact that the extracts from the roots delayed the germination of seeds of mustard and cress.

The dormancy of tuberous perennating organs has been investigated extensively at the Boyce Thompson Institute. In a paper by N. C. Thornton (1945) the opinion was expressed that the low level of metabolism in stored potatoes was due to the accumulation of metabolic by-products which are not oxidized in the normal way because of the low oxygen-supply to the inner tissues resulting from the slow diffusion of the gas through bulky tissues. Recent publications by W. G. Burton (1950 and 1951) from which the data in Table I were taken indicate, however, that although the oxygen concentration

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in the intercellular spaces of potatoes is lower than in air the difference (only 8 per cent. lower when stored at 25° C.) is not very great and would hardly be expected to have much effect on respiratory oxidation processes according to data quoted by James (1953). Only when the oxygen concentration falls below 5 per cent. does the respiratory quotient begin to rise, indicating the commencement of anaerobic respiration. Burton's data show, however, that the internal atmosphere in potatoes contains a very considerable concentration of carbon dioxide amounting to 7 per cent. for tubers stored at 25° C. These considerations suggested to the author that possibly the formation of salt-absorption inhibitor was a result of the increased internal carbon-dioxide concentration which, although it has not been measured for beetroot, is likely to be as great as in potatoes under comparable conditions of storage.

TABLE I

Analysis of the Gas in the Intercellular Spaces of Potatoes

(Data from W. G. Burton 1950 and 1951)

Storage temperature	Composition of the internal atmosphere	
	O_2%	CO_2%
5° C.	18·1	2·6
10° C.	18·2	1·8
15° C.	16·8	3·9
20° C.	16·1	4·7
25° C.	13·0	7·0

The depressing effect of carbon dioxide on various metabolic processes has been reported by many authors. F. Kidd (1914) showed that 12 per cent. carbon dioxide induced a condition of prolonged dormancy in imbibed seeds of *Brassica alba* which could only be broken by drying the seeds, by removal of the tests, or by exposure to chloroform vapour. H. Burstrom, in a private communication to the author, has reported that carbon dioxide prevents the active absorption of water by the roots of wheat plants grown in culture solution.

Skelding and Rees (1952) showed that tissue discs newly cut from stored beetroot, after absorbing manganese rapidly for a few hours, almost ceased to absorb further salt for a period of 50–70 hours before a steady rate of absorption was established. The initial phase of rapid absorption was attributed to cationic exchange between the cytoplasm and the external medium so that it may be said that in dormant tissue there is a 'lag' of 50–70 hours before metabolic absorption commences. A similar lag had been reported previously by W. Stiles (1924) who found that its duration could be reduced by washing the tissue in running tap water before placing in the salt solutions. This lag period could conceivably be due to the natural inhibitor present in the stored tissues. If this were the case, however, the suppression of the metabolic absorption of manganese could hardly be due to the presence of carbon dioxide as such in solution in the cell sap, since this would be removed within a very short time of cutting the tissue into slices only 1 mm. thick. The continuation of the lag for some 60 hours would suggest that the active inhibitor was a combined form of carbon dioxide. That this is not an unreasonable speculation is shown by the numerous reports of the fixation of carbon dioxide in the dark, which have appeared in the literature since Werkman and Wood discovered the process in proprionic bacteria in 1942. Hill and Whittingham (1955) in Chapter 6 of their book *Photosynthesis* state that 'assimilation of CO_2 has been shown in the dark in all the main groups of living organisms including plants'. In the same chapter they give an account of numerous enzyme systems extracted from a wide variety of organisms which are capable of fixing carbon dioxide *in vitro* in a number of different types of chemical combination. M. Thomas (1949) reviewed the knowledge of the acid metabolism of succulent plants in the light of Wood and Werkman's discovery and reported new work by Thomas and Beevers (1949) and Thomas and Ranson (published later in 1954), which showed that the leaves of a range of succulent species and a few which were not succulent would fix carbon dioxide in the dark in the form of malic acid and yield it up to photosynthetic assimilation on exposure to light.

In view of the succulent nature of the genus *Beta* and the bulky nature of the root it seemed worthwhile to examine the possibility that the inhibition effect on salt absorption might be due to some substance formed by dark fixation of carbon dioxide. The results of this investigation are reported below.

EXPERIMENTAL METHODS AND MATERIALS

Plant material. The beetroot used was a round rooted variety 'Sutton's Crimson Globe', grown in the University of Birmingham Botanic Garden. The roots were harvested at the end of October and stored in a large crate filled with moist sand in a fruit room of which the temperature varied between 6 and 12° C. through the winter. Plugs of tissue were taken from roots with a 20 mm. cork-borer and these were cut by hand microtome into discs 1 mm. thick. The outer layer of tissue to a depth of 1 cm. below the periderm was always rejected so that discs were all cut from the more deeply seated cells. Discs were normally washed for 20 minutes in running tap water to remove materials released from the cut cells before commencing the experimental treatment. Such short periods of washing were found to have no noticeable effect on the natural inhibition of salt absorption. In some experiments prolonged washing periods were used deliberately to reduce the natural inhibitor content. The discs were threaded on silver wire spaced with glass beads.

Solutions. The tissue absorbed manganese from solutions of manganous chloride of approximately 0·001 M concentration. The ratio of the number of discs to the volume of solution varied according to the requirements of the particular experiment from 5 discs in 20 ml. of solution to 20 discs in 200 ml. A number of different arrangements were used at different times to provide for the aeration of the tissue. When large volumes of solution were used (160 or 200 ml.) these were contained in wide-necked jars of about 420 ml. capacity fitted with ground-in glass lids perforated by a hole 1 cm. in diameter.

These were agitated in a reciprocating shaker at a rate of 1 cycle per second with a 3-inch throw. Smaller volumes (20 ml.) of solution were agitated in $1\frac{1}{2}$-inch-diameter 'Pyrex' boiling-tubes at about 3 cycles per second with a $2\frac{1}{2}$-inch throw. Further particulars of some of the special treatments used are given in the detailed consideration of the experimental series.

The solutions were maintained in water baths thermostatically controlled at $21 \pm 0{\cdot}5^\circ$ C. or in a constant temperature room at $21 \pm 1^\circ$ C.

Manganese analysis. The absorption of manganese was calculated from the polarographic analysis of 5 ml. samples of the solutions taken firstly between 3 and 6 hours from the beginning of the experiment and at approximately 24-hour intervals thereafter. After each sample if necessary the manganese concentration was restored to 0·001 M by adding 0·02 M $MnCl_2$ from a burette and the solution made up to the original volume with distilled water. In experiments in which only 20 ml. of solution were used, however, the solution was replaced completely after each sample.

Presentation of the results. The unit which has been used in all cases to express the amounts of manganese absorbed is the microgramme-molecule (micromol or μM), viz. the molecular weight in grammes $\times 10^{-6}$. This is a convenient size for the quantities involved. For expressing the total amount of manganese absorbed during an experiment all the results have been reduced to a basis of 1 cubic centimetre of tissue. In earlier experiments the author has used both fresh weights and dry weights as units of tissue and found that the variability of the data was in no way reduced. The present unit was therefore adopted as saving a considerable amount of labour with no loss of precision. Absorption-rates have also been used in the presentation of the results. For these a tissue unit of 1 c.c. proved too small when rates were calculated on an hourly basis, so a unit of 100 c.c. was adopted instead. Absorption-rates are all given therefore in μM per 100 c.c. of tissue per hour.

The experimental results are given in graphical form throughout. In the first series of experiments in which carbon dioxide was present the whole time it was thought desirable to plot the mean manganese absorption rate for the interval between two samples against the central time for the interval, progressively through the experiment. The points have been joined by straight lines. Data of this kind are normally represented by means of histograms but these have not been used as they give confusing pictures when a number of curves are required on the same set of axes. As most of the quantitative comparisons made are between different treatments within the same experiment, samples being taken simultaneously, the error involved by joining up the points plotted in the way described is not likely to be serious. In the second series of experiments it was necessary to compare the course of recovery of the tissue from preliminary treatments with carbon dioxide and this is better shown by graphing the total manganese absorbed from the beginning of the experiment up to the time of sampling against time, progressively through the experiment. The same method of presentation was also adopted in the third experimental series.

The experimental results are conveniently considered under the following three headings:

I. The effect of the presence of carbon dioxide on manganese absorption.

II. The effect of a preliminary treatment with carbon dioxide on the subsequent absorption of manganese.

III. The effect of gas and high temperature storage of beetroot on the manganese absorption of the tissues.

I. THE EFFECT OF THE PRESENCE OF CARBON DIOXIDE ON MANGANESE ABSORPTION

In the majority of experiments in this series samples of 5 discs were immersed in 25 ml. of solution in boiling-tubes of 125-ml. capacity. The atmosphere in the tubes was replaced with the experimental gas mixtures by passing a rapid stream (20 litres per hour) through the solution for 30 minutes twice daily after which the tubes were tightly stoppered and agitated at 3 cycles per second. The accumulation of respiratory carbon dioxide caused a change in the composition of the atmosphere inside the tubes during the course of the experiments, but as beetroot discs respire about 1·5 mg. CO_2 per 10 g. fresh weight per hour the greatest possible increase in CO_2 concentration before the gas and solutions were renewed was only about 1·5 per cent. Gas mixtures were obtained by mixing streams from gas cylinders measured through calibrated capillary flow meters.

Experiment 1. *The effect of* 10 *per cent. carbon dioxide on manganese absorption*

The two gas mixtures used were 10 per cent. CO_2+90 per cent. air and as the control 10 per cent. nitrogen+90 per cent. air. The experiment was performed in October using new beetroot which, because they were taken directly from the ground, had probably a low inhibitor content. To make this even more certain they were washed for 12 hours before beginning the experiment. The absorption-rates plotted on a time-scale are shown in Fig. 1. The rate of manganese absorption under carbon-dioxide treatment was a little over one-half that of the control for the first 100 hours of the experiment. Although the control-rate declined more rapidly than the CO_2-rate after this time the total absorption by the end of the experiment was 35 per cent. lower in CO_2. The marked decline in the rate of absorption in the control treatment seems to be due to a limited capacity for manganese absorption which has been noted on other occasions by the author in roots newly lifted from the soil. The control approaches this limit sooner than the CO_2 treated and suffers a sharper decline in consequence. Expt. 1 showed that a concentration of carbon dioxide, which it is reasonable to suppose may exist within a large storage root, produces a marked effect on manganese absorption by the cells of the tissue. In Expt. 2 the effect of a much greater concentration was investigated.

Experiment 2. The effect of carbon dioxide at high concentrations

In this case the manganese-chloride solution was saturated with CO_2 by passing the gas through the stock of solution for 30 minutes before measuring out the 25 c.c. quantities into the boiling-tubes to which the discs were added. The controls consisted of manganese chloride solution in equilibrium with air. A measured sample of the solution containing CO_2 was added to a measured quantity of standard potassium hydroxide solution and the residual hydroxide titrated with standard acid. The CO_2 concentration was found to be 1·99 g. per litre of solution which is only slightly less than the quantity that will dissolve in water at 20° C. and normal pressure. After measuring out 25 ml. aliquots of the solution into boiling-tubes they were shaken for 30 minutes in an uncorked condition before closure to ensure that air was present inside. Loss of CO_2 during this time and by subsequent equilibration with the air in the tubes after closure reduced the concentration to which the discs were exposed to a value possibly as low as one-third of saturation, a fact which was reflected in the rise of the pH of the solutions from 3·65 at the beginning to about 5 after 24 hours. As, however, the solutions were replaced from the stock after each sample (24 hours) the discs must have been exposed to an average CO_2 concentration of at least 40 per cent., whilst they were absorbing manganese. In view of the low pH of the carbon-dioxide-containing solution it was thought necessary to investigate the possibility that the anticipated reduction of manganese absorption might be due to the acidity. Consequently, a third treatment was introduced in which the manganese chloride was acidified with hydrochloric acid to a pH value of 3·54. The pH values of all the solutions were measured at the beginning and end of each

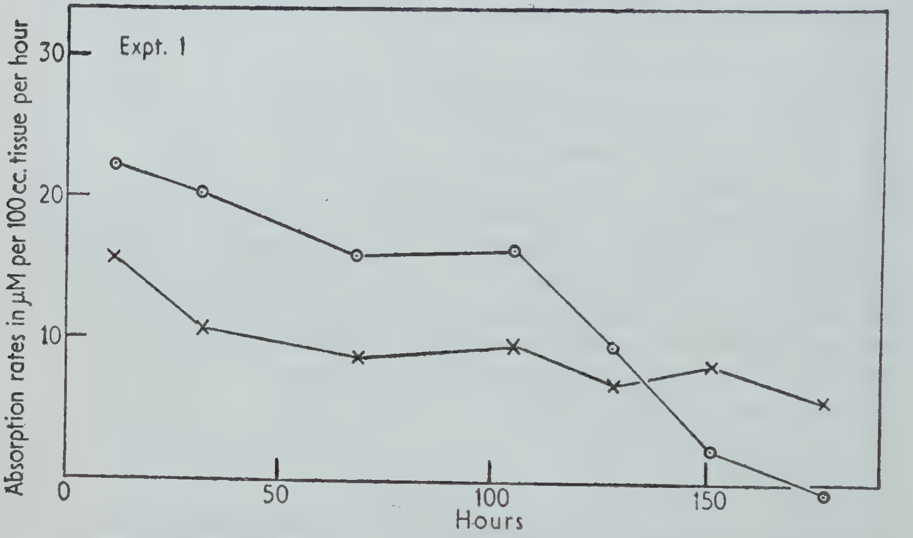

FIG. 1. Rates of manganese absorption by red beet tissue from 0·001 M solutions of manganese chloride in the presence of 10% carbon dioxide; × CO_2; ⊙ control.

24-hour period of absorption. The results are shown in Fig. 2. It will be seen that the absorption-rate was low at the beginning of the experiment and approximately the same in all treatments. (The first rate on the graph is the average value during the first 20 hours of the experiment and includes a component which is due to rapid cation exchange when the discs were first placed in the solutions. It is, therefore, abnormally high and may be disregarded.) The low initial rate is explicable by the presence of natural inhibitor because the experiment was made at the end of January after about 3 months' storage during which inhibitor forms in the roots. As the tissue was subjected to only a short preliminary washing much of this would still be present in the cells at the beginning of the experiment. In the presence of high CO_2 concentrations this low initial-absorption-rate persisted almost unchanged for 150 hours whereas when no CO_2 was present it rose steadily throughout the experiment to five times the value. The presence of hydrochloric acid had no effect on the rising absorption-rate for 100 hours although after this a levelling out of the curve occurred. Exposure to the high CO_2 concentration did not seem to produce injury of the tissue since an immediate rise in the absorption-rate resulted from changing over the treatments after 150 hours. We may conclude that the depression of manganese absorption caused by CO_2 is not due to the acid reaction which it promotes. Expts. 1 and 2 have indicated that the higher the concentration of carbon dioxide the greater the degree to which manganese absorption is depressed. Expt. 3 was designed therefore to establish more fully the relationship between the concentration of the gas and the reduction of salt absorption.

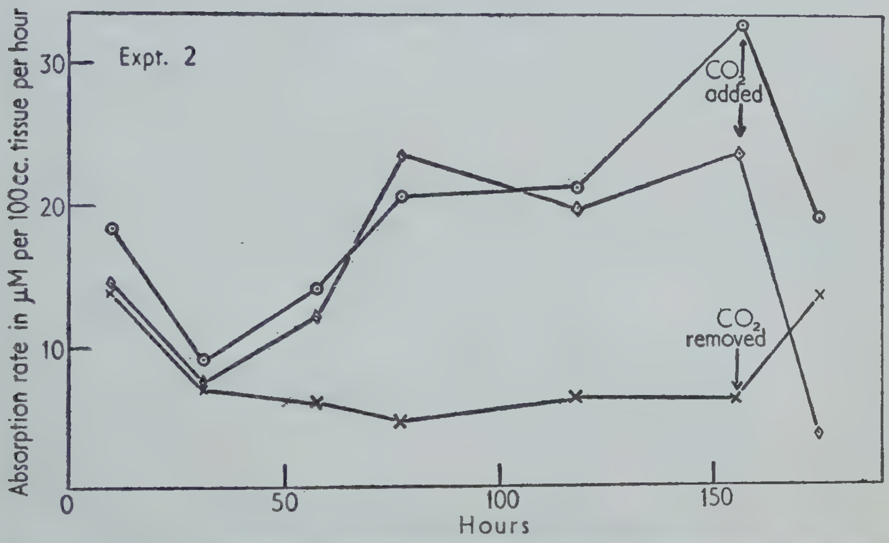

FIG. 2. Rates of manganese absorption by red beet tissue from solutions saturated with carbon dioxide and from solutions containing hydrochloric acid having the same pH value; × CO_2; ◇ HCl; ⊙ control.

Experiment 3. *The effect of carbon dioxide concentration on the absorption of manganese ions*

The experimental arrangements were similar to those adopted in Expt. 1. Five discs of beetroot tissue were placed in 25 ml. of solution contained in boiling-tubes through which gas mixtures were passed for 30 minutes twice daily and which were then sealed and placed in the shaker. The experiment was performed at the end of January when the natural inhibitor content of the roots was at its greatest and as the washing period after cutting was of only 30 minutes' duration it was expected that the initial rate of manganese absorption would be low. The solutions were aerated with the six different gas mixtures listed below, each treatment being duplicated. In all except one treatment oxygen was used as the complement of the carbon dioxide in the mixtures to avoid the possibility of the reduction of the absorption-rate by oxygen deficiency in the higher concentrations.

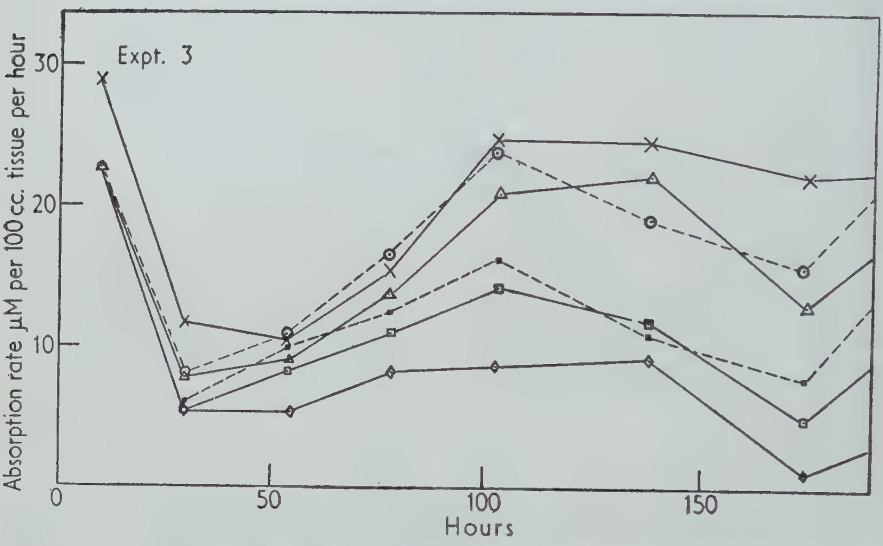

FIG. 3. Rates of manganese absorption by red beet tissue in the presence of various concentrations of carbon dioxide; × 3·3% CO_2; △ 10%; ☐ 23·5%; ◇ 33%; ⊙ oxygen; ■ air.

A. 3·3% CO_2+complement of oxygen
B. 10·2% CO_2
C. 23·5% CO_2
D. 33% CO_2
E. Oxygen alone—free from CO_2
F. Air—free from CO_2

The results as absorption-rates are shown in Fig. 3. It will be seen that the absorption-rates are at first low but follow a rising course during the first 100 hours which is presumed to be due to removal of natural inhibitor from the tissue. Subsequently the rate declined slowly though somewhat irregularly. During the first 100 hours the rising curve of absorption-rate was similar in 3·3 per cent. CO_2 and in the CO_2 free controls, but during the next 170 hours the rate in oxygen showed a distinct decline whilst that in 3·3 per cent. CO_2 maintained a steady level. Both the rates and the total accumulated absorption after 100 hours were significantly greater with 3·3 per cent. CO_2 than with the control. Higher CO_2 concentrations reduced the absorption-rate progressively. Fig. 4, in which the average absorption-rate between 100 and 150 hours is plotted against CO_2 concentration, shows a linear relationship between these quantities. It may be predicted by extrapolation that the complete suppression of absorption would result in about 50 per cent. CO_2. Forty per cent. CO_2 would produce an absorption-rate approximately the same as that shown by freshly cut tissue. As each point on the graph represents the mean of only two rates for each of two replicates, however, the linear relationship between absorption-rate and carbon-dioxide concentration is probably only approximate and no great importance is attached to exact linearity in this paper. There is a possibility that the reduced manganese absorption by discs of beetroot in the presence of CO_2 may be due to the lower oxygen concentrations which result unavoidably from the use of higher CO_2 concentrations. A comparison of the absorption-rates for oxygen and air in Fig. 3 shows that the maximum rates in air are about 30 per cent. lower than in oxygen. If the effect of reduced oxygen concentration on manganese absorption is approximately linear over the range of concentration between 100 and 21 per cent. then the effect of using a 33 per cent. carbon dioxide-oxygen mixture would have caused no greater a reduction than 13 per cent. attributable to lowered oxygen tension, leaving a reduction of at least 50 per cent. due to carbon dioxide.

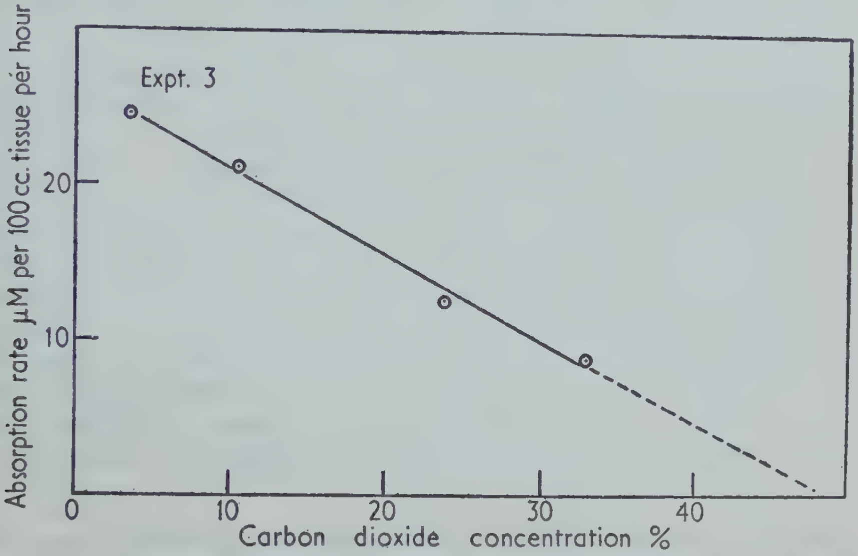

FIG. 4. The relationship between carbon-dioxide concentration and the rate of manganese absorption by red beet tissue.

The experiments so far described have demonstrated a direct effect of carbon dioxide on the absorption of manganese ions by metabolic processes in beetroot tissue. The magnitude of the reduction in absorption-rate is apparently related to the concentration of the gas to which the tissues are exposed up to a limit of about 40–50 per cent. beyond which value there is little further reduction. The characteristic rising curve of absorption-rate with time shown by tissue freshly cut from beetroot in the middle of the winter-storage period is progressively suppressed by increasing concentration of carbon dioxide, so that a concentration of between 40 and 50 per cent. might be expected to maintain the low initial rates. This fact suggests the possible similarity between the natural condition of stored beetroot tissue and the condition which may be induced by exposure to substantial concentrations of carbon dioxide with regard to its capacity for manganese absorption. Taken in conjunction with the evidence cited by Skelding and Rees (1952) for the presence of a salt-absorption inhibitor in stored beetroot the results of the experiments given above appear to support the hypothesis that the natural inhibitor might well be a carbon-dioxide fixation product.

In the experiments described so far, however, the inhibition of salt absorption only persisted as long as the tissue was exposed to CO_2. When the gas was replaced by air or oxygen free from carbon dioxide increases in the absorption-rate began immediately whereas in naturally inhibited tissue it was normally between 50 and 70 hours after cutting before the absorption-rate began to increase. If it were possible to demonstrate that the CO_2 pre-treatment of tissue with a low natural-inhibitor content induced a 'lag' period in salt absorption similar to that shown by freshly cut stored tissue, this would provide even stronger support for the formation of inhibitor from carbon dioxide. This was attempted in the second series of experiments.

II. THE EFFECT OF PRELIMINARY TREATMENT WITH CARBON DIOXIDE ON THE SUBSEQUENT ABSORPTION OF MANGANESE

In the experiments of this series discs of beetroot tissue, after periods of washing which varied according to the requirements of the occasion, were placed in 2-litre flasks containing the appropriate gas mixtures. These mixtures were renewed twice daily and the flasks sealed at other times. In the flasks used for carbon-dioxide-free control treatments small waxed tubes containing 40 per cent. potassium hydroxide and rolls of filter paper were included to remove respiratory CO_2. After this pre-treatment the tissue was transferred to manganese solutions in boiling-tubes or jars in the shaker as in the previous series.

Experiment 4. *The effect of pre-treatment with* 10 *per cent.* CO_2 *in air*

The experiment was performed in early October on newly lifted roots in the expectation that there would be a low inhibitor content. To reduce this further the discs were given 27 hours' preliminary washing. The duration of pre-treatment with 10 per cent. CO_2 or 10 per cent. N_2 using air as a filler, was 44 hours. Fig. 5 showing the total absorption of manganese shows a significantly longer lag period after carbon-dioxide pre-treatment than in the controls. It also shows that when the lag period is over the absorption rate following CO_2 treatment declines less rapidly than the controls in much the same way as the 3·3 per cent. and 10 per cent. carbon-dioxide treatments in Expt. 3. Whilst this could be taken as an indication of a stimulation effect following the removal of inhibitor it may merely be due to the tissue having a limited capacity for manganese absorption since the CO_2 pre-treated discs never absorb more manganese than the controls. The observation that both absorption curves are distinctly flatter after 200 hours would also favour this explanation. This result of the experiment, though a small one, was sufficient to encourage further investigation and in Expt. 5 the effect of varying the duration of pre-treatment was examined.

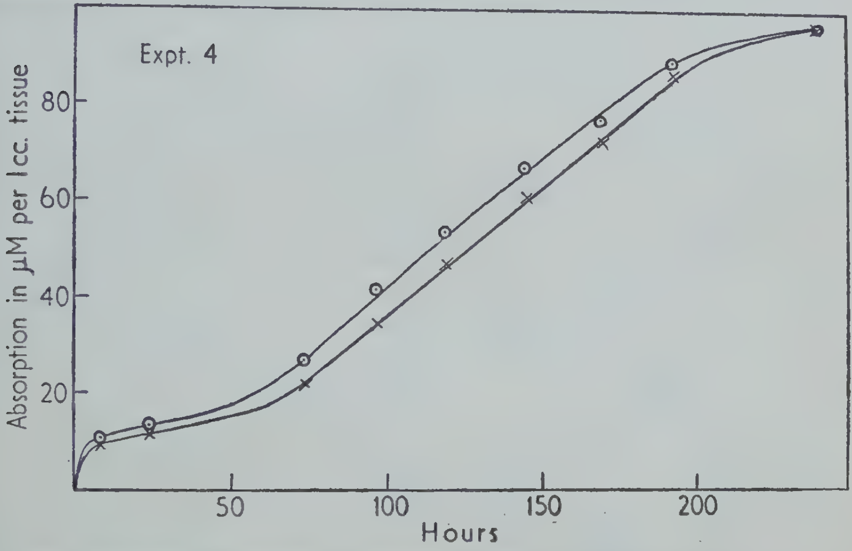

FIG. 5. The course of absorption of manganese by red beet tissue following pre-treatment with 10% carbon dioxide; × CO_2; ⊙ control.

Experiment 5. *The effect of increasing duration of pre-treatment with* 10 *per cent. carbon dioxide*

The method was that of the previous experiment except that oxygen was used as a filler instead of air in view of the fact that the stimulation of absorption by 3·3 and 10 per cent. CO_2 in Expt. 3 occurred when oxygen was used. Pre-treatments of 24, 48, 72, and 96 hours were given. The experiment was performed in early November and further to ensure a low initial inhibitor level the discs were washed for 96 hours before exposing them to CO_2. The low level of inhibitor is confirmed by the very short lag period shown after the shortest pre-treatment. Fig. 6 shows that 48 hours' pre-treatment with 10 per cent. carbon dioxide produced a stimulation of salt absorption which was statistically significant. A slight stimulation, too small to be significant, was found after 24 hours' pre-treatment. Ninety-six hours' pre-treatment with CO_2 produced a significant depression of the manganese absorption and a smaller depression (not significant in this experiment) was found after 72 hours. There appears therefore to be a dual effect of carbon dioxide, short exposures producing a stimulatory effect and long exposures the opposite. As a similar change of effect with increasing concentration of carbon dioxide was found in Expt. 3 the effect of increasing concentration of carbon dioxide in pre-treatment was investigated in Expt. 6.

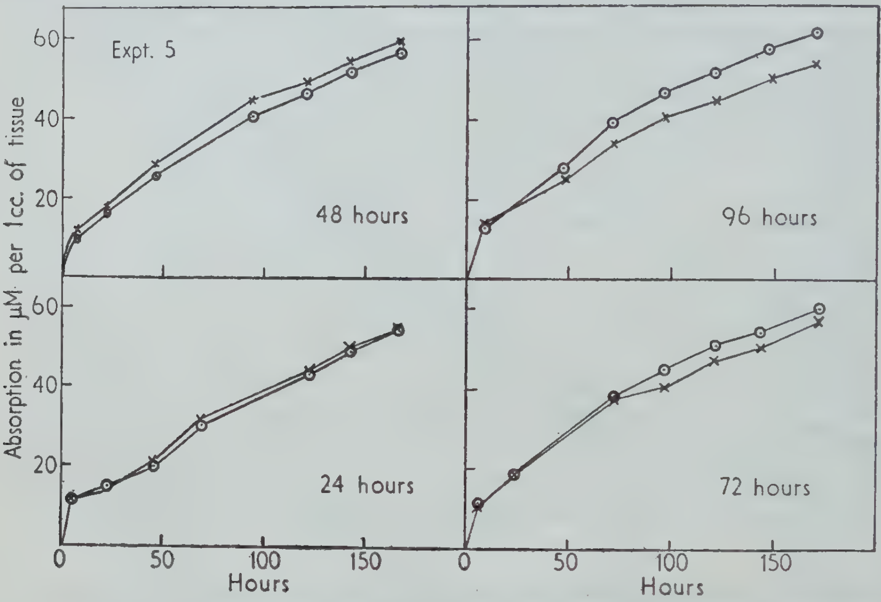

FIG. 6. The course of manganese absorption by red beet tissue following pre-treatments of 24, 48, 72, and 96 hours with 10% carbon dioxide; × CO_2; ⊙ control.

Experiment 6. *The effect of pre-treatment with different concentrations of carbon dioxide*

The experiment was performed at the end of November after a storage period of about 1 month. The sliced tissue was washed for 24 hours, followed by 43 hours' pre-treatment with 5, 15, or 30 per cent. carbon dioxide, using oxygen as the filler. Fig. 7 shows a slight (not significant) stimulation produced by 5 per cent. CO_2 and a significant depression after treatment with 15 and 30 per cent. CO_2. There was very little difference between the effects of 15 and 30 per cent. The latter result appears to conflict with Expt. 3 in which the absorption-rate showed a proportional fall with CO_2 concentration up to 33 per cent. A valid comparison would be between the rates of absorption after 43 hours (the pre-treatment time in Expt. 6) in the earlier experiment and the initial rates in Expt. 6. In Expt. 3, however, the low initial absorption-rates of newly cut tissue had hardly yet begun to rise at the forty-third hour of carbon-dioxide treatment. A close comparison of the two experiments is not possible for a number of reasons, however. In Expt. 6

carbon dioxide was only present during pre-treatment whilst in Expt. 3 it was present throughout the experiment. In Expt. 3 the gas was in aqueous solution whilst in Expt. 6 it was in gaseous form. Finally in Expt. 3 the tissue had a high natural inhibitor content whereas that of Expt. 6 was initially very low.

Comparing the result of Expt. 6 with that of Expt. 5 the absence of a stimulatory effect of 5 per cent. carbon dioxide, such as was produced by 48 hours' exposure to 10 per cent. in Expt. 5, may be explained by the lower concentration of gas acting for a shorter time.

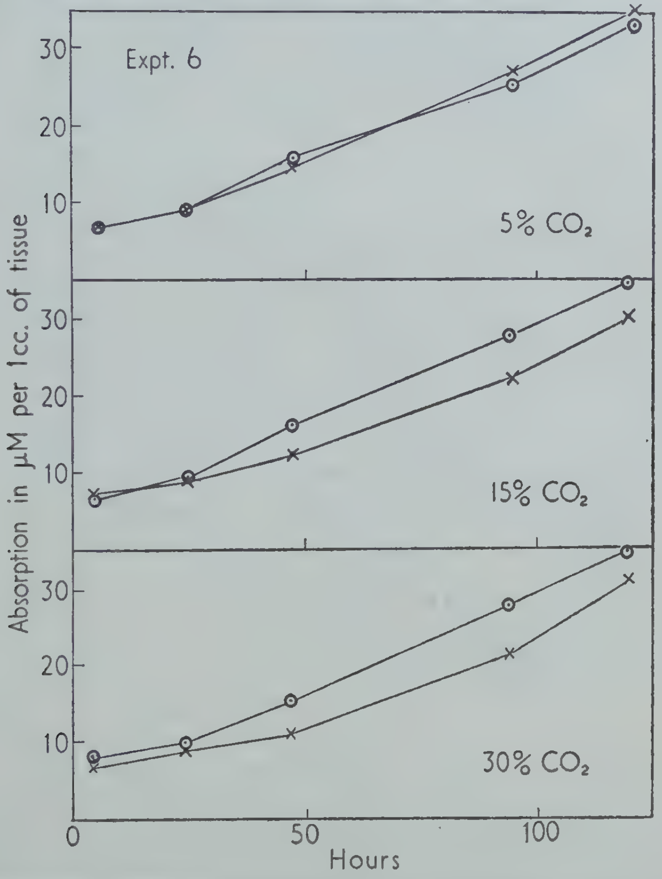

FIG. 7. The course of manganese absorption by red beet tissue following pre-treatment for 43 hours with 5%, 15%, and 30% carbon dioxide; × CO_2; ⊙ controls.

The foregoing experiments have shown that carbon-dioxide pre-treatment of tissue with a low level of natural inhibitor can reduce its capacity for manganese absorption for some time after the gas is removed and that the time curves of recovery from this condition resemble that of recovery from the condition found in tissue newly cut from stored roots. If the natural inhibitor is a combined form of carbon dioxide it should be possible by exposure to fairly high concentrations of CO_2 to maintain or increase the natural inhibitor content. A preliminary experiment which is not reported in detail showed that 4½ days' exposure to 20 per cent. carbon dioxide with air as the complement was insufficient to maintain the degree of inhibition found in freshly cut beetroot in the middle of January. The lag period of the freshly cut tissue was about 80 hours' duration, that of tissue treated for 4½ days in 20 per cent. CO_2 only about 50 hours and that of the control tissue (20 per cent. N_2) reduced to about 25 hours.

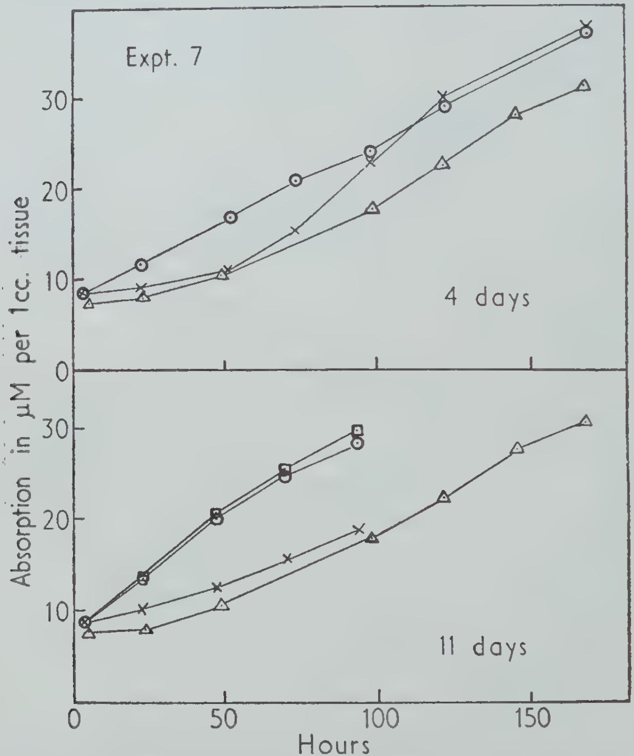

FIG. 8. The course of manganese absorption by red beet tissue following 4- and 11-day pretreatments with 26% carbon dioxide; × CO_2; ⊙ control; △ newly cut tissue; ⊡ tissue pretreated for 11 days in running water.

Experiment 7. The effect of prolonged pre-treatment with 26 per cent. carbon dioxide in oxygen on the removal of natural inhibitor

The tissue, cut from beetroot at the end of January and washed for 2 hours only, had a high content of natural inhibitor. This was confirmed by the absorption/time curve for discs set to absorb manganese immediately after washing which showed a lag period lasting 70 hours. Other samples were treated with 26 per cent. CO_2+74 per cent. oxygen or 26 per cent. N_2+74 per cent. oxygen for 4 days and for 11 days and others were washed in running tap water for 11 days. Fig. 8 shows manganese accumulation following these treatments. A concentration of 26 per cent. CO_2 maintains the initial absorption rates (during the first 50 hours after transferring to manganese solutions) at a level almost as low as that of newly cut tissue for as long as 11 days. In fact it is possible that the inhibitor content of the fresh tissue is increased somewhat. The control tissue and the sample washed for 11 days in running water showed removal of the inhibitor probably reaching completion before 11 days' pre-treatment, since the initial absorption-rate on placing in manganese was then at a maximum. After 4 days' CO_2 pre-treatment the absorption-rate of manganese was at first no greater than that of the freshly cut tissue but after 50 hours in the solutions the rate began to increase and soon became significantly greater than the controls. Here again as in Expts. 3 and 5 we have evidence of the stimulation of salt absorption associated with exposure to carbon dioxide in the presence of high oxygen concentrations. The fact that no stimulation was found after 11 days' pre-treatment may be due to the stopping of the experiment after 96 hours but even if a stimulation had developed after this time it would have been delayed in comparison with the 4-day pre-treatment.

A hypothesis suggested here to interpret these facts is that the fixation of carbon dioxide in beetroot tissue may give rise to two compounds, one inhibitory in its effect on manganese absorption and the other stimulatory. The inhibitor may be formed under low oxygen concentrations such as air but the stimulant formation seems only to occur appreciably in abnormally high oxygen concentrations. In Expt. 7 it may be supposed that during pre-treatment the total inhibitor content of the tissues increased by further CO_2 fixation but at the same time owing to high oxygen concentration some stimulant formation also occurred. The effect of the stimulant was masked by the inhibitor on first placing the tissue into manganese solutions but became apparent after the removal of the inhibitor which always occurs when tissue is placed in water. The greater inhibitor concentration after 11 days' pre-treatment with CO_2 might be expected to mask the stimulation effect for the rather short duration of the experiment.

From the foregoing experiments it seems reasonable to conclude that the natural inhibitor of salt absorption present in whole stored beetroot may be lost after cutting by two processes operating simultaneously, namely by reversal of the carbon dioxide fixation process when the external partial pressure of the gas is reduced and by some oxidation of the inhibitor especially when the oxygen pressure is high. To these must be added the solution of the inhibitor when the tissue is washed in water since concentrated aqueous extracts of beetroot possess an inhibitory action. If both fixation reversal and oxidation are in fact operative it should be possible to remove inhibitor from tissue before cutting into discs by the application of suitable pre-treatments. The results of such an experiment are given below.

Experiment 8. *The removal of inhibitor by pre-treatment of tissue in bulk*

The experiment was performed at the end of February when the natural inhibitor content was still fairly great. Cylindrical plugs 2 cm. in diameter and 7 cm. long were taken from one large beetroot and, after 1 hour's washing in running water, placed in litre flasks. In two of these the air was displaced by either oxygen or nitrogen by passing a rapid stream of gas through for 15 minutes, after which they were sealed. The third flask was evacuated to a pressure of 30 mm. of mercury and then sealed. The treatments were renewed twice daily for 2 days. The seal of the evacuated flask was imperfect and the vacuum always fell to about 500 mm. of mercury by the time for re-evacuation.

After treatment the plugs were cut into discs, washed for 15 minutes and set to absorb manganese. The results given in Fig. 9 show that evacuation and oxygen treatment were both more effective than nitrogen in removing the inhibitor. Evacuation was more effective than treatment with oxygen but this was to be expected as oxidation of the inhibitor is certain to occur as air re-entered the tissues in addition to its removal due to the rapid extraction of carbon dioxide from the intercellular spaces of the tissue.

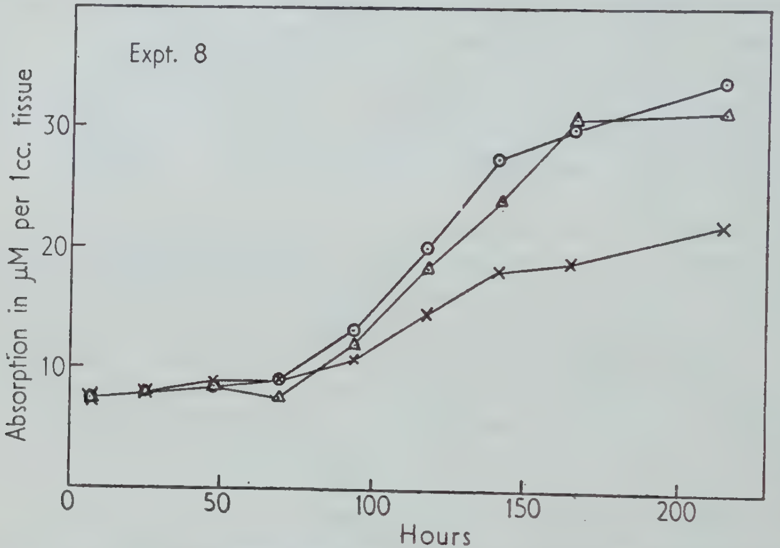

FIG. 9. The course of manganese absorption by red beet tissue following pre-treatment (before cutting into discs) with oxygen, nitrogen, and by evacuation; △ oxygen; × nitrogen; ⊙ evacuation.

III. THE EFFECT OF GAS AND HIGH TEMPERATURE STORAGE OF BEETROOT ON THE MANGANESE ABSORPTION OF THE TISSUES

M. B. Zaman (1953) found no inhibition of manganese absorption in beetroot newly raised from the ground in October. Using the length of the lag period before the commencement of metabolic absorption as a measure of the inhibitor content of roots, he found that inhibitor was formed during storage, reached a maximum concentration in the tissues about the end of January and then began to decline until sprouting roots in May had none. If carbon-dioxide fixation is the source of inhibitor as the experiments described above suggest, its formation should be accelerated by storage at raised temperatures or in closed containers which would increase the CO_2 concentrations within the tissues. In October 1951 freshly raised roots were kept under the following conditions:

1. In pots of moist sand in cool store at temperatures between 6°–12° C.
2. In pots of moist sand in greenhouse at temperatures between 22°–27° C.
3. In large sealed jars at temperatures of 22°–27° C.

Roots from treatment 2 were sampled after 6 weeks' storage and roots from all treatments after 10 weeks and the course of manganese absorption compared with that of the newly raised roots. The results are shown in Fig. 10.

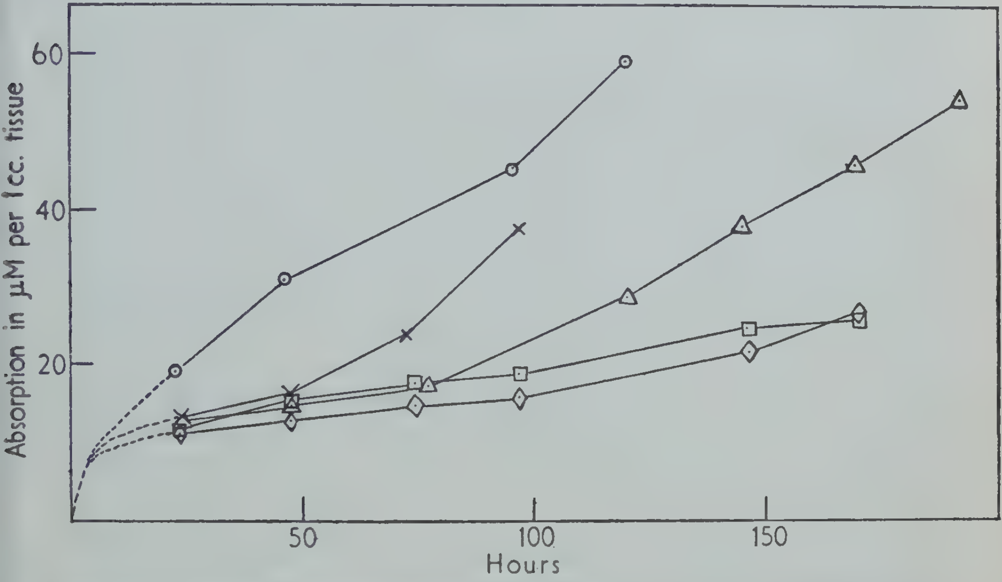

Fig. 10. The course of manganese absorption by red beet tissue from roots stored at high and low temperature, in closed or open conditions and for various durations; ⊙ roots newly raised from ground; × roots stored 10 weeks at 6°–12° C.; △ roots stored 6 weeks at 22°–27° C.; ⊡ roots stored 10 weeks at 22°–27° C. in open conditions; ◇ roots stored 10 weeks at 22°–27° C. in enclosed conditions.

The newly raised beetroot show no lag period and a high rate of metabolic absorption. After 10 weeks' storage at 6°–12° C. a lag period lasting 55 hours had developed. After 10 weeks' storage at 22°–27° C. the length of the lag period could not be estimated because the original low rate of absorption showed no tendency to increase during the 150 hours that the experiment lasted. Fig. 10 in which a comparison is made between roots stored at 22°–27° C. in open and enclosed conditions shows significantly less absorption by tissue treated in the latter way. It might be thought that the continued low absorption-rate after storage at 22°–27° C. implied permanent injury to the tissues although there was no abnormal loss of red pigment. The curve for roots stored for 6 weeks at 22°–27° C., however, shows a lag period lasting nearly twice as long as for those stored for 10 weeks at 6°–12° C. but nevertheless once the lag period was over a normal rate of metabolic absorption developed indicating that 6 weeks' exposure to 22°–27° C. had no injurious effect. It is evident then that storage under higher temperatures and under enclosed conditions such that respiratory carbon dioxide cannot escape both accelerate the formation of inhibitor.

DISCUSSION

In the experiments described above it has been proved that carbon dioxide in concentrations similar to those which have been shown by other workers to occur naturally in the intercellular spaces and so also in solution in the tissues of bulky storage organs, has the effect of lowering appreciably the capacity of beetroot tissue to absorb manganese ions from solutions. Tissue slices newly cut from beetroot in the middle of the winter storage season similarly have a low capacity for manganese absorption and it is not unreasonable to suppose that the reason for this may be associated with the effect of the accumulated respiratory carbon dioxide to which they have been exposed for many weeks inside the relatively impermeable periderm of the root. Supporting this view are the findings of the second experimental series that tissue slices freed from the naturally inhibited condition by prolonged washing in running water lose, to varying degrees, the capacity for the immediate absorption of manganese ions after exposure to gaseous carbon dioxide. The extent of this carbon dioxide induced inhibition and its duration after removal from the gas depend roughly upon the concentration of CO_2 which has been used and upon the duration of the exposure. So far as the author is aware this effect of carbon dioxide upon metabolic salt accumulation has not been reported previously although a depressant effect of CO_2 on other fundamental physiological processes, such as respiration, growth, and seed germination, have been known for a long time.

The hypothesis is advanced here that the inhibiting substance, the existence of which in beetroot was established by the author jointly with Dr. W. J. Rees in an earlier paper, is probably formed by carbon dioxide fixation. Fixation has not been proved for beetroot in this series of experiments but so many instances of dark fixation by plant tissues have been cited in physiological journals during recent years that it is highly probable that an internal accumulation of CO_2 would result in the process occurring at least to some extent. Most of the better-known enzyme systems concerned with carbon-dioxide fixation have been shown to be reversible, although *in vitro* the equilibrium is usually in the direction of decarboxylation at CO_2 concentrations of the order of 5–10 per cent. as for example the isocitric decarboxylation system described by Whatley (1951). If, however, such enzyme systems are coupled with dehydrogenase systems or if the activity of the primary product of fixation is reduced by combination with some other cell constituent, then the balance of the reaction will be in favour of carboxylation. In an instance due to Ochoa for animal tissue quoted by Hill and Whittingham (1955) the fixation of CO_2 to form malic acid was approximately half saturated at a carbon-dioxide concentration of 17 per cent. when the system was coupled with glucose-6-phosphate dehydrogenase. The carbon-dioxide inhibition of manganese accumulation is also reversible according to the concentration to which the tissue is exposed, and it is also worth noting that maximum inhibition occurred with concentrations of the gas of about 40 per cent.—i.e. roughly twice the value which Ochoa found for half saturation of the pyruvic-malic fixation system. The results of the experiments reported here seem therefore to agree fairly well with the hypothesis of the inhibition of salt accumulation by a product of carbon-dioxide fixation although the final proof of its origin necessarily involves the isolation and chemical characterization of the inhibiting substance and the demonstration that it could be formed by carbon-dioxide fixation. Further work is proceeding on these lines.

The loss of inhibitor by tissue freshly cut from stored roots can apparently take place by reversal of the fixation reaction and also, since aqueous extracts of stored beetroot tissue were shown in earlier work to contain an inhibiting principle, by solution in water.

It also seems likely from the results described here that a third method of inhibitor removal may exist. In Expts. 3, 5, and 7 low concentrations of carbon dioxide or short durations of exposure in tissue initially free from inhibitor produced a stimulatory effect on manganese accumulation, after an initial inhibition had disappeared. The effect was most noticeable (Expts. 3 and 5) when oxygen was the complementary gas. This suggests the possibility of an oxidative removal of the inhibitor in addition to a decarboxylative one. The conversion of growth inhibiting substance to growth stimulants was referred to by Evanari (1949) in his review although oxidative processes were not mentioned. The question of the nature of this stimulant action following inhibition, however, needs further investigation.

Finally, in the third series of experiments it has been shown that the degree of natural inhibition of manganese absorption by beetroot, as judged by the length of the lag-period between cutting the discs and the commencement of metabolic absorption, is related to the conditions of prior storage in a way which might be expected if inhibitor is formed by CO_2 fixation. Inhibition was shown to develop more rapidly at higher storage temperatures which cause more rapid production of respiratory carbon dioxide and also under enclosed conditions when the gas is unable to escape.

SUMMARY

1. It has been shown that the presence of carbon dioxide caused a depression in the rate at which discs of beetroot tissue absorb manganese from 0·001 M solutions of manganese chloride. The effect was not caused by the acidity produced by the carbon dioxide in solution, nor by oxygen deficiency, nor by permanent injury of the cells.

2. Manganese absorption-rates were inversely proportional to the external

carbon-dioxide concentration up to a maximum of about 40 per cent., which produced minimal absorption-rates of about 5μM of Mn/100 c.c. tissue/hour.

3. Carbon-dioxide concentrations of about 3 per cent. produced a stimulation of manganese absorption compared with the controls when the complementary gas was oxygen, although the effect did not appear until about 100 hours from the commencement of the treatment.

4. Preliminary exposure of well-washed beetroot discs to atmospheres containing carbon dioxide produced a decreased capacity for manganese absorption (the lag phase) lasting for about 50 hours after placing in solutions. This condition resembled that of tissue newly excised from beetroot in the middle of the winter storage season, attributed in an earlier publication to the presence of an inhibiting substance. The possibility exists therefore that the inhibitor is formed by a dark fixation of carbon dioxide.

5. The duration of the lag phase in manganese absorption increased with increasing concentration of carbon dioxide when air was the filler gas up to 15 per cent. CO_2 but not appreciably above this value in a pre-treatment lasting 48 hours. The duration of the natural lag phase in newly cut beetroot tissue was, however, considerably increased by 11 days' exposure to 26 per cent. CO_2 when oxygen was the filler gas. After 4 days' pre-treatment with this mixture, once the lag phase of manganese absorption had been completed, a stimulation of absorption-rate compared with that of the controls was found. A similar stimulation resulted from pre-treatment of well-washed tissue (i.e. containing no natural inhibitor) for 48 hours with 10 per cent. CO_2+90 per cent. oxygen, although 96 hours' exposure to this mixture depressed manganese absorption.

6. The hypothesis suggested here to explain the experimental data is that a substance capable of inhibiting manganese absorption is formed by the dark fixation of some of the respiratory carbon dioxide which is known to accumulate in the intercellular spaces of bulky storage tissues. Many examples of the dark fixation of carbon dioxide by plant tissues have been reported. The removal of this inhibitor in cut tissues may be by diffusion from the cells into water during washing, by reversal of the fixation process when the partial pressure of carbon dioxide is lowered, and possibly also by oxidative conversion into a substance capable of stimulating manganese absorption when the external oxygen concentration is high.

7. In addition to the results cited above, the greatly increased inhibition of manganese absorption following storage at high temperatures and in enclosed containers, both of which have the effect of increasing the carbon dioxide concentration in the tissues, lends support to the hypothesis, final proof of which must depend on identification of the inhibitor and the establishment of its origin from carbon dioxide.

ACKNOWLEDGEMENTS

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Observations on the Cytology of *Halidrys siliquosa* (L.) Lyngb.

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With Plates V–VIII

Abstract

A study of the cytology of *Halidrys siliquosa* suggests that it has a haploid thallus with reduction division immediately following fertilization. There appear to be eight chromosomes in vegetative cells and developing antheridial and oogonial cells. Typical 'bouquet' and diakinesis stages suggesting a reduction division have been seen on germination of the zygote.

Introduction

IN a paper read before the Royal Society on 18 June 1896, Farmer and Lloyd Williams gave the first description of the nuclear cycle in the Fucales. Most of their observations had been made on *Fucus vesiculosus*, *Fucus spiralis*, and *Ascophyllum nodosum*. They had shown that in the vegetative cells of each of them, 'the chromosomes when assembled on the spindle, at the equator, are seen to be twice as numerous as in the oogonial nuclei. Hence it follows that the reduction in the number of chromosomes (in the female plants) is associated with the differentiation of the oogonium—the mother cell of the sexual products.' They stated that in spite of repeated attempts they had not succeeded in observing the first nuclear division in the oogonium. It was at the two succeeding divisions resulting in the formation of the eight oospheres, that they had counted the haploid number of chromosomes. After fertilization, they claimed to have seen the diploid number at the first division of the oospore. Again, in Strasburger's account (1897) of *Fucus serratus*, it was inferred, but not actually observed, that reduction in the number of chromosomes takes place during differentiation of the oospheres. It was more than ten years later that Yamanouchi (1909) demonstrated that the first nuclear divisions in both the oogonium and the antheridium of *Fucus vesiculosus* were meiotic. Since the publication of this detailed account, no further observations of the actual reduction division in any member of the Fucales have been made, as far as we are aware.

In their paper of 1896 Farmer and Williams commented on the extreme small size of the chromosomes and the difficulties experienced in counting them. They estimated the number in the vegetative cells of *Fucus* sp. as twenty, while the oogonial nuclei had the haploid number of ten. Two years later the same authors, working with exactly the same species, gave the diploid number as twenty-eight. Strasburger (1897) had meanwhile given the diploid

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number for *Fucus serratus* as thirty, but Farmer and Williams were inclined to place it somewhat lower; approximately twenty-six to twenty-eight. There is an enormous difference between these numbers and Yamanouchi's 2n = sixty-four for *F. vesiculosus* (1909). One may ask, can such great variations be attributed only to difficulties of observation? Or was Yamanouchi's material, collected from Wood's Hole, U.S.A., of a different generic strain from the same species collected from the eastern shores of the Atlantic by Farmer and Williams?

Nienburg (1910) has described nuclear division in the oogonium of *Cystoseira barbata* Ag. and was convinced that at the first division he saw a typical synapsis stage, although he did not succeed in counting the actual number of chromosomes at the following metaphase. At the third nuclear division in the oogonium he counted eighteen to twenty chromosomes, which he believed to be half the number of those he saw during vegetative divisions. Nuclear divisions in the antheridia were not studied.

Several papers on the cytology of Japanese members of the Fucales have appeared, such as those of Inoh (1935) and Shimotomai (1928). Counts were made of the number of chromosomes in the oogonial nuclei of *Pelvetia Wrightii* (Yendo), *Fucus evanescens* Ag., and *Cystophyllum sisymbriodes* J. Ag. respectively. Although no counts were made at all on the somatic nuclei of any of these species, the number counted in the oogonia was assumed to be the haploid number. Thus in the published literature there is very little detailed description of nuclear behaviour during the life cycle of members of the Fucales.

MATERIAL AND METHODS

Unless otherwise stated, all the plants of *Halidrys siliquosa* used for this investigation were collected from a population in the shallow rock pools at mid-tide level on the eastern side of St. Mary's Island, Northumberland.

The investigation was started in October, when no dividing nuclei could be found in either the vegetative tips of the plants, or in the receptacles. Farmer and Williams (1898) had remarked that their best results had been obtained from plants which had been collected from a boat 2–3 hours after the tide had advanced and covered them. As Yamanouchi (1909) had also commented on the tidal effects on nuclear division, we fixed portions of apices and receptacles at intervals of high and low tide throughout 24-hour periods, but still no dividing nuclei could be found. By repeating fixations at weekly intervals during a complete year, it became apparent that the time of day and the state of the tide had no effect whatever on nuclear divisions of *Halidrys*. The essential point revealed was that a short period of vegetative growth took place in May and June when the meristematic regions were full of dividing nuclei. This was followed by the development of the reproductive organs in which nuclear divisions were delayed until just prior to the shedding of the gametes during the winter.

We experimented with numerous fixatives including Bouin, Karpechenko, and Carnoy made up in both distilled water and sea-water. We finally selected Langlet, made up in distilled water, as giving the best results. The formula is

A.	B.
Chromic acid 1g.	40% formalin 40 ml.
Glacial acetic acid 10 ml.	Distilled water 35 ml.
Distilled water 65 ml.	

Equal quantities of solutions A and B are mixed prior to fixation.

For the study of somatic nuclei, sections were cut at 4–6 μ, while for gametic nuclei and for the developing oospores thicker sections at 7–12 μ were necessary. They were stained in either Heidenhaim's haematoxylin or gentian violet. Aceto-carmine smears were very successful on the antheridia.

SOMATIC NUCLEI

(*a*) *Vegetative apices*

As previously stated, the vegetative apices grew rapidly during May and June, and material fixed at any time of the day was full of dividing nuclei in both the meristoderm and the mass of meristematic segments cut off immediately beneath the apical cell. At other times of the year the apices were resting and the nuclei took up none or very little stain. The resting nuclei are small and generally each one has a large and very conspicuous nucleolus.

At the onset of prophase densely staining particles become visible, as though scattered over the surface of the nucleus. At late prophase the chromosomes were well distributed throughout the nucleus and could be easily counted. At metaphase, with all the fixatives with which we experimented, there was a tendency for them to clump together.

In the somatic cells the chromosomes can be distinguished easily from one another. There are eight present in each nucleus, three of which are longer than the rest and generally appear V-shaped (Fig. 1, Plate V). One of the chromosomes has often been observed to be either hooked or with a satellite. There is always one chromosome conspicuously smaller than the rest. Divisions are simultaneous throughout the apical meristematic tissue, but in the meristoderm, all phases of nuclear division can be found within short distance of each other.

A narrow-leaved variety, *H. siliquosa* var. *siliculosus* was distinguished by Newton (1931) and it is synonymous with the var. *gracilis* of Holmes and Batters and the var. *minor* of Stackhouse. During a biological study of the genus it became apparent that considerable variation in thallus width occurred, and that as well as narrow-leaved plants agreeing with descriptions of the above variety, an extremely wide form was found. Consequently plants at the two extremes of thallus width: a narrow form from Beadnell, Northumberland and a wide-leaved form from Salcombe, Devon, were examined. No difference in chromosome number between the 'varieties' was observed, and the somatic cells of both forms contained eight chromosomes similar in form to those described in plants from St. Mary's Island.

(*b*) *Sterile hairs of the conceptacles*

Conceptacles were first recognizable on the plants towards the end of July. They continued to develop over the next few months and could be found on plants till the following May. Active growth of the sterile paraphyses took place during August and September, before the maturation of the gametes. Counts of chromosomes gave the number as eight, and they were similar in form to those observed in the cells of the vegetative apices (Fig. 2, Plate V).

(*c*) *Antheridial nuclei*

In spite of the fact that conceptacles were differentiated as early as July, antheridia could not be distinguished until the end of August or beginning of September. The antheridia were differentiated from the tips of paraphyses. A minute cell, which subsequently developed into the first antheridium, was cut off at the apex. Beneath it a protrusion grew out which again became cut off from the paraphysis by a transverse wall. In a similar way a succession of antheridia were produced around the first formed one, so giving rise to a tuft of four or more at the tip of each paraphysis. At first the antheridia were uninucleate, and for several weeks the nuclei were resting and showed no staining either with gentian violet or aceto-carmine. The first dividing nuclei were found at the beginning of December and from that time onwards antheridial development proceeded for several months.

It was the first division in the antheridium in which we were most interested, as it was here that Yamanouchi saw bivalents and other features associated with a meiotic division, in *Fucus vesiculosus*. In *Halidrys* the antheridial nucleus enlarges prior to division, and in early prophase densely staining granules again become conspicuous, as in the prophase of somatic nuclei. In late prophase of the first division (Fig. 3, Plate V) it was possible to distinguish eight distinct chromosomes well dispersed throughout the nucleus. They were similar in form to those observed in the somatic nuclei, the three larger V-shaped ones being conspicuous. Again at the second division eight chromosomes were clearly differentiated. At the third and fourth divisions it was still possible to count eight chromosomes in some of the nuclei. At succeeding divisions the chromosomes were so small that we found it impossible to determine the number with any accuracy. However it is clear that no reduction in chromosome number has taken place up to the fourth division in the antheridium.

(*d*) *Oogonial nuclei*

The young oogonia can first be recognized as enlarged cells of the conceptacle wall, projecting slightly into the conceptacle cavity. The whole cell contents were dense and granular, and about the centre of the cell is a comparatively large nucleus having the appearance of a hollow vesicle with densely staining granules scattered over the surface. A single large and conspicuous nucleolus is always present. Often associated with the nucleolus are one or several refractive granules, resembling very much the figures of 'intra-nucleolar chromosomes' described by Svedelius (1937) in the red alga, *Lomentaria rosea*. From August to October the oogonia enlarge considerably but the nuclei remain the same size. They rest during this time, lose their granular appearance, and absorb little or no stain.

Division of the oogonial nucleus is delayed until just prior to gamete discharge. Then the divisions must be completed within a very short time, for we had great difficulty in finding them; indeed, we observed them only in one collection of material which had been fixed *in situ* on St. Mary's Island on an exceptionally cold morning of January, when a layer of ice had first of all to be removed from the pools where the plants were growing. We tried fixations at hourly intervals throughout 24-hour periods, first at ordinary room temperature and then following refrigeration, but never again did we succeed in obtaining dividing nuclei.

At the onset of division the single oogonial nucleus after its long resting period assumes a granular appearance, resembling the beginning of prophase of somatic nuclei. At metaphase, side view, of this first division we were able to count eight chromosomes as shown in Fig. 4 where they are beginning to divide and to move to opposite poles. They were of distinctive shape, and not contracted as one would expect in a meiotic division. During this division the nucleolus persists well into metaphase. The diameter of the two daughter nuclei of the division is approximately half that of the parent nucleus. Each one of the daughter nuclei moves to opposite sides of the oogonium and then undergoes another division. Fig. 5 shows a late prophase of this second division, again with eight distinct chromosomes visible. Although at this stage the chromosomes are very small, the three large V-shaped ones can still be distinguished. Thus during this division there has been no reduction in chromosome numbers.

The four daughter nuclei which result are very small and are dispersed towards the periphery of the cytoplasm. We did not observe a third division giving rise to eight egg nuclei as has been described for the oogonia of many of the Fucales. Three of the four nuclei moved to the periphery of the oogonium and by the time of maturity they are excluded from the cytoplasm of the oosphere. Meanwhile the remaining nucleus enlarges and takes up a central position in the cytoplasm. Within a short time of shedding, deep staining granules cluster around this remaining nucleus and render it difficult to determine.

(*e*) *Fertilization and oospore development*

In order to study the behaviour of the oogonial nucleus after fertilization sporelings were cultured in the following manner. A number of ripe receptacles were placed in petri-dishes containing fresh sea-water and left in a cool greenhouse for approximately 24 hours. A gradual discharge of eggs and antherozoids took place, although there were many more discharged during the night than during the day. *Halidrys* is hermaphrodite and both male and female gametes mature simultaneously in the same conceptacle. At the end of one day the receptacles were removed from the dish, and in the area surrounding where each one had been, would be the mass of discharged eggs and sperms. If left untouched for a further day the fertilized eggs would adhere to the bottom of the dish and the sea-water could then be changed frequently without disturbing them. During the first 4 days after discharge eggs were fixed at frequent intervals. Sporelings could be maintained in these culture dishes for several months.

Fertilization and the subsequent germination of *Halidrys*' eggs is slow. A fertilization membrane did not form around the eggs until about the second day after they had been shed from the conceptacles. By this time the egg nucleus was still in about the centre of the cell, and the sperm nucleus had moved towards it. In Fig. 9, Plate VIII, the two nuclei are shown prior to their fusion. They are of approximately similar size and each has one very prominent nucleolus. Each nucleus has the appearance of a hollow vesicle surrounded by a delicate network of chromatin, the whole being enclosed in a very distinct nuclear membrane. After fusion (Fig. 10, Plate VIII) the resulting nucleus is approximately twice the diameter of each sperm and egg nucleus, and it contains two conspicuous nucleoli, presumably one having been derived from each gamete. The fusion nucleus has a fine chromatin network dispersed over its surface, and it is surrounded by a very thin nuclear membrane. The granules surrounding the egg nucleus are then repelled so that the fusion nucleus is surrounded by a 'halo' of clear cytoplasm fringed with deeply staining granules. The fusion of the egg and sperm nuclei takes place when both nuclei are in an early prophase. The fusion nucleus then undergoes what we believe to be a reduction division.

During the early stages of division the chromatin network becomes a tangled mass of threads which thicken, and often aggregate to one side of the nucleus forming a typical bouquet stage of a meiotic division. Pairing of the threads, with the formation of chiasmata, then follows as in Fig. 7 (Plate VII). Here the nucleus has flattened laterally, thus becoming more oval in shape instead of its previous spherical form. Asters and centrioles are conspicuous at this stage, such as we were not able to observe in any of the previous divisions we have already described. Judging from the number of nuclei which can be found in this stage of division in material fixed from 40 hours to 4 days after gamete extrusion, this phase lasts for a considerable time. It is followed by diakinesis, when the paired chromosomes shorten and thicken (Fig. 8, Plate VIII). Here there are eight pairs of chromosomes dispersed throughout the nucleus and with the nuclear membrane still intact. At a slightly later stage when the membrane is broken down, it is difficult to determine the number of bivalent chromosomes with any accuracy, owing to the proximity of deeply staining granules in the adjacent cytoplasm.

During metaphase the nucleus shrinks in size, and eventually the paired chromosomes arrange themselves on the equatorial region of a small spindle, which is intra-nuclear in origin. The paired chromosomes are shortened and no longer distinctly recognizable as in the divisions of somatic nuclei and of those in the antheridia and oogonia. During anaphase the spindle is drawn out lengthwise, so that at telophase the two groups of chromosomes lie at opposite sides of the oospore. The reconstituted nuclei are approximately half the size of the fusion nucleus. A septum then advances from the periphery towards the centre of the cell (Fig. 6, Plate VI) eventually cleaving the protoplast into two, with each half containing one of the daughter nuclei. Nuclear divisions then proceed rapidly and the ratio of nucleus to cell size increases, all while the cells are still contained within the fertilization membrane. Later the sporeling elongates and differentiation of tissues begins. In sporelings several weeks old it is possible again to distinguish the eight chromosomes characteristic of the somatic cells of the adult thallus.

(*f*) *Abnormalities in chromosome number*

Amongst the cultures of sporelings we frequently came across eggs showing abnormally high chromosome numbers at the first metaphase. Plates of 16, 32, and 40 chromosomes were sometimes found. As we remarked earlier, we did not find it practicable to count the chromosomes in polar views of metaphase plates, owing to the tendency of the chromosomes to clump. Yet here, with these high numbers the chromosomes did not show the characteristic clumping. Nor were they paired as in the normal reduction division we have just described. How do such high numbers originate? And what is their fate?

Farmer and Lloyd Williams (1898) had recorded instances of polyspermy in *Fucus* sp. and it may be that here in *Halidrys* we have the entry and fusion of several sperms with the egg nucleus. Alternatively, none, or only some of the oogonial nuclei may degenerate, so that several of them fuse with one sperm nucleus. The fact that we have found most of these high numbers of chromosomes in metaphase plates suggests that there may be a halt in the nuclear division here and probably most of them develop no further.

Discussion

The presence of such a low number as eight chromosomes renders the study of the somatic divisions of *Halidrys* comparatively easy. The chromosomes too have distinctive shapes which have been recognized in cells behind the apical meristem, in the meristoderm, and in the sterile paraphyses of the conceptacles. The complement of eight chromosomes, amongst which it is possible to distinguish three larger ones, usually V-shaped, one chromosome conspicuously smaller than the rest, and only one with a satellite, suggest that these are the somatic set of a haploid individual and not of a diploid.

This is the first recorded attempt to determine the chromosome number of so called 'varieties' of a brown seaweed. The same number of chromosomes was found in 'normal' plants from St. Mary's Island, Northumberland, a wide-leaved 'variety' from Salcombe, Devon, and a narrow-leaved 'variety' from Beadnell, Northumberland. A subsequent statistical analysis of plants showed a continuous variation from broad- to narrow-leaved forms suggesting that all of them should be regarded as within the same species, and not as distinct varieties (Lacey, 1956).

The divisions which were observed in both the antheridia and oogonia were similar to those in vegetative cells. It was possible in both to distinguish the same number of eight distinct chromosomes. If reduction took place during gamete formation as Yamanouchi (1909) described in *Fucus vesiculosus*, then the first division in both the antheridia and the oogonia should have shown 4 pairs of chromosomes with only 4 chromosomes at subsequent divisions, instead of the 8 which was counted. The observation that reduction division did not occur in either the oogonium or the antheridium of *Halidrys* suggests two alternatives: either the eggs develop parthenogenetically, or reduction division takes place on germination of the zygote.

The antherozoids of *Halidrys* are certainly attracted to the eggs, and although we never actually saw an antherozoid penetrate and enter an egg, the illustration in Fig. 9, Plate VIII, shows the two nuclei prior to fusion. One nucleus lies slightly above the other, and hence in the photograph neither of them are in sharp focus. Both nuclei are in early prophase, and are of approximately similar size. After fusion, the resulting nucleus (Fig. 10, Plate VIII) is twice the size of each fusion nucleus, and is still in early prophase. These observations rule out the possibility of the first alternative of parthenogenesis.

On germination of the oospore typical bouquet, pachytene and diakenesis stages of a reduction division have been observed. In the latter, the eight chromosomes are obviously paired and shortened, typical of a reduction division. This means that *Halidrys* is a haploid organism, the only diploid phase being represented by the zygote, with reduction division taking place on germination. Such a life cycle occurs in many of the Chlorophyta designated by Baker (1955) as dimorphic haplonts. The zygote after meiotic division gives rise to 1, 2, or 4 haploid spores, which on germination give rise to thalli similar to those bearing gametes, but in *Halidrys* the diploid zygote is giving rise directly to a new haploid thallus.

SUMMARY

1. In cells of the meristoderm, of the apical meristematic tissue and of the sterile paraphyses, a complement of eight chromosomes has been determined.

2. In the nuclear divisions of the antheridium it has been possible to distinguish the same number of chromosomes as in the somatic cells.

3. Only two nuclear divisions take place during development of the oogonium, and at each of these divisions eight chromosomes have been counted.

4. Typical 'bouquet' and diakinesis stages of a reduction division have been seen on germination of the zygote.

5. The evidence suggests that *Halidrys siliquosa* has a haploid thallus, with reduction division occurring immediately following fertilization.

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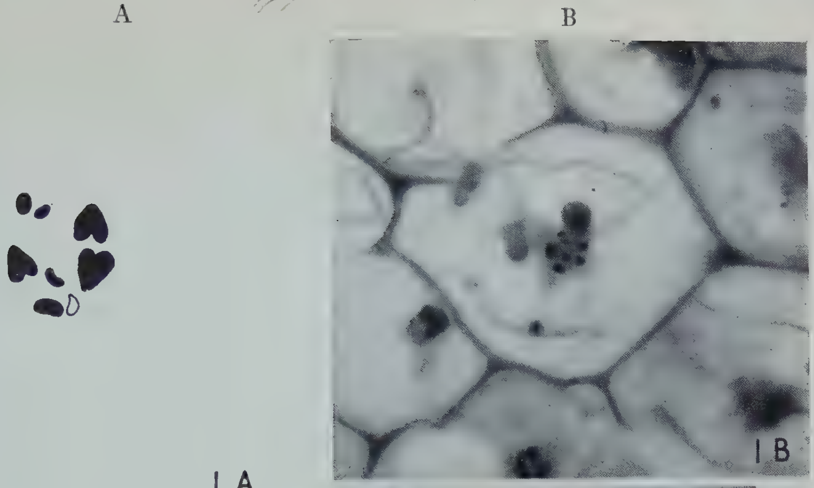

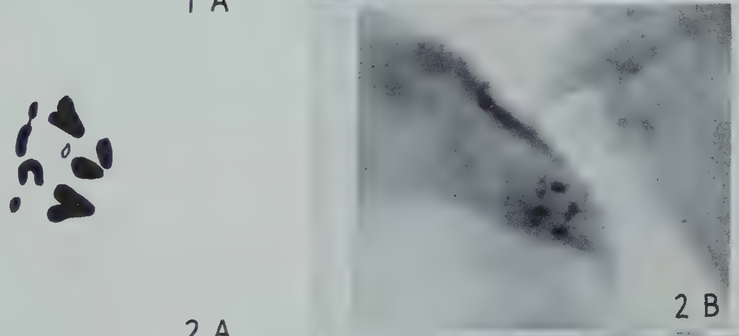

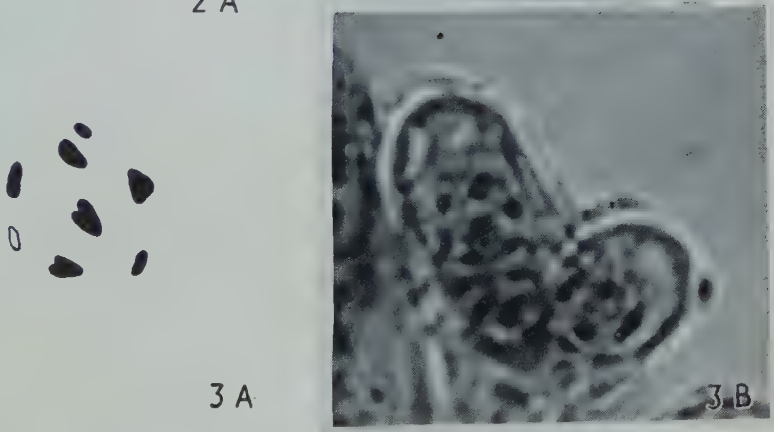

FIG. 1. Chromosomes of a vegetative cell behind the apex of *Halidrys siliquosa*. In the photograph only seven of the eight chromosomes are visible; the eighth (shown in the drawing) is at a lower focal level

A × approximately 3800

B × approximately 1400

FIG. 2. Late prophase chromosomes in a paraphysis of *Halidrys siliquosa*. The small centrally placed chromosome is out of focus in the photograph

A × approximately 3800

B × approximately 1400

FIG. 3. Late prophase of the first nuclear division in an antheridium. Only the shadow of the eighth chromosome is visible in the photograph

A × approximately 3800

B × approximately 1400

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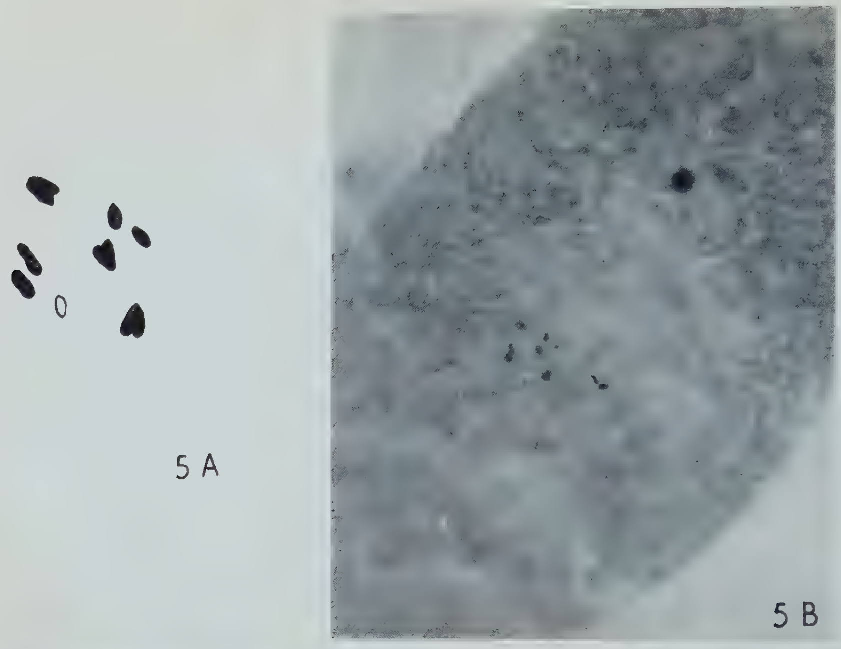

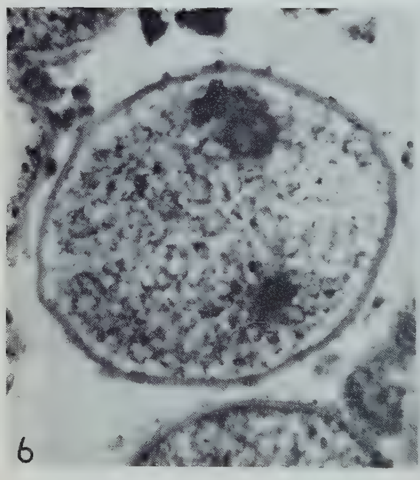

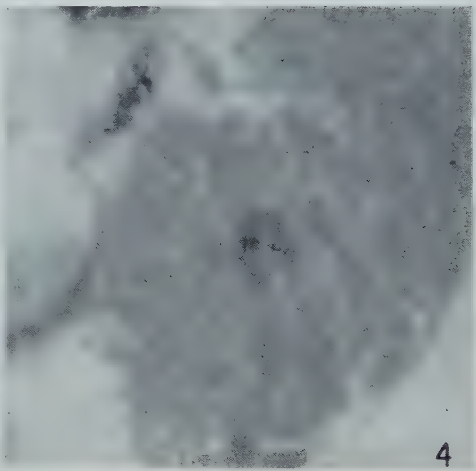

FIG. 4. Side view of the metaphase of the first nuclear division of the oogonium, showing approximately seven of the eight chromosomes, two of which have already divided.
× approximately 800

FIG. 5. Late prophase of the second nuclear division in the oogonium. Only seven of the eight chromosomes are in focus in the photograph of the lower nucleus, while only the nucleolus is visible in the upper one
A × approximately 3800
B × approximately 1400

FIG. 6. Septum formation commencing to advance from the periphery across the cytoplasm after completion of the first nuclear division in the oospore
× approximately 500

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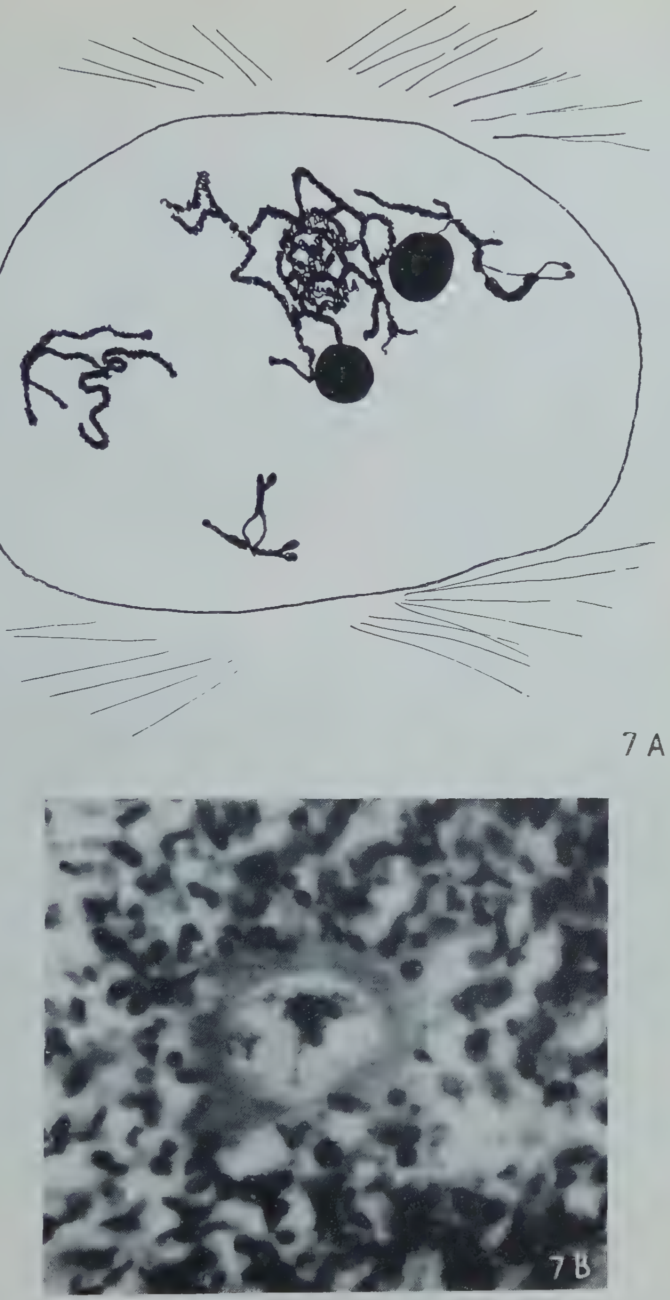

Fig. 7. First division of the nucleus following fertilization. The drawing of the nucleus photographed below shows two prominent nucleoli and pairing of some of the chromosomes. Faint rays of the aster are also visible in the photograph

A × approximately 3800
B × approximately 1000

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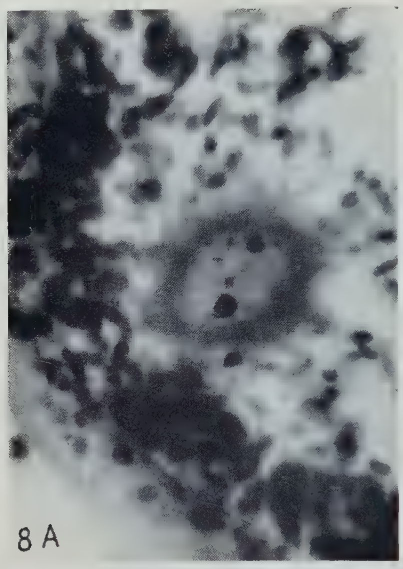

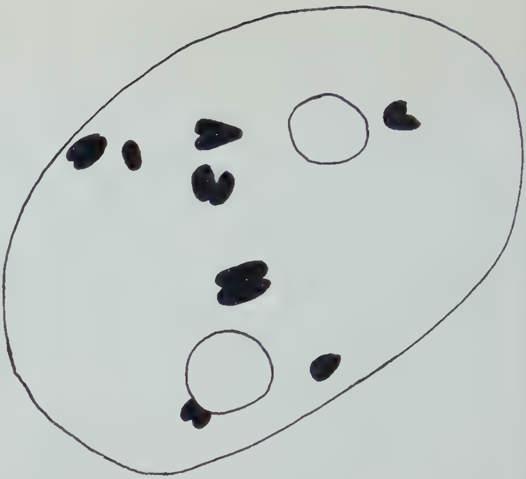

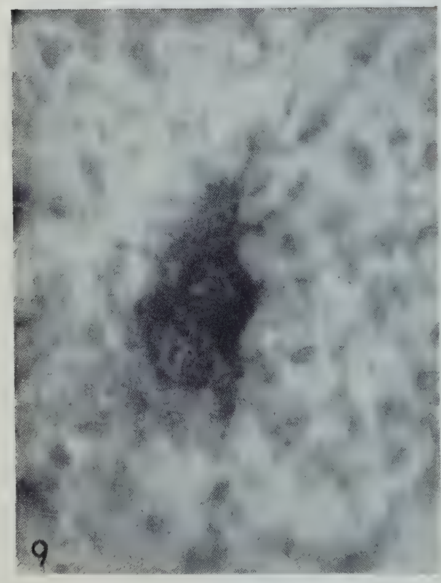

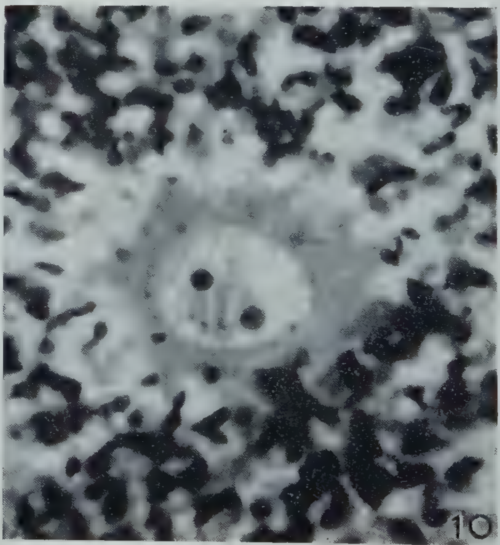

FIG. 8. Late prophase of the first division following fertilization. Two prominent nucleoli are visible in the photograph as well as some of the paired chromosomes which are enlarged in the drawing

FIGS. Fertilization of the egg

9. The two nuclei prior to fusion. One nucleus lies slightly above the other so that neither of them is in sharp focus

10. After fusion, showing the nucleus with two conspicuous nucleoli and a fine network of chromatin Both × approximately 1000

FIGS. 8A, 9, and 10 approximately × 1000.

FIG. 8B approximately × 3800

B. L. MOSS *and* E. ELLIOT

Nutritional Factors Influencing the Production of Sclerotia by Certain Fungi

BY

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ABSTRACT

In various fungi (*Botrytis cinerea*, *B. allii*, *Sclerotinia gladioli*, *Sclerotium cepivorum*, *S. rolfsii*, and *Rhizoctonia solani*) the initiation of sclerotia, their further development to full size and their maturation were three distinct stages with different nutritional requirements. These processes were followed on peptone-salts media with a variety of carbon sources (carbohydrate or mannitol) ranging in concentration between 0·5 and 3 per cent. The nitrogen nutrition was varied in the presence of 2 per cent. glucose with peptone replaced by asparagine, potassium nitrate, or ammonium salts. All were suitable provided a high acidity did not develop. Urea was unsatisfactory. Details given include the rate of sugar utilization in some examples.

INTRODUCTION

THE importance of sclerotia as a means of survival of plant parasitic fungi is well known. The factors which lead to their formation, however, are not fully understood. Few species have been investigated in detail, but the results available show that, in general, sclerotia are produced most readily by conditions favouring mycelial growth, but that their requirements of water, food, &c., are greater than those of minimum mycelial growth. The available data have recently been reviewed by Hawker (1956).

MATERIALS AND METHODS

The following fungi were studied: *Botrytis cinerea*, *B. allii*, *Sclerotinia gladioli*, *Sclerotium rolfsii*, *S. cepivorum*, and *Rhizoctonia solani*. Stock cultures of these were maintained on malt agar slopes. In experimental work the fungi were grown on agar media in petri-dishes of 9 cm. diameter, each of which contained 30 ml. medium, or in liquid media in conical flasks containing 30 ml. medium per flask.

The rate of increase of diameter of the colony was taken as an indication of the amount of mycelial growth. This was not an absolute measure of growth, owing to the fact that it disregarded the density of the hyphal mat, but it usually revealed distinctive features. During the early stages of this work, the method of recording the sclerotial production was by inspection and an arbitrary classification. Exact estimation was not practicable with the sclerotia of *S. gladioli* and *S. cepivorum* which were very small, whilst those of *R. solani* could not be separated from the mycelium and substratum.

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In later experiments, the relatively large sclerotia of *B. cinerea*, *B. allii*, and *S. rolfsii* were estimated by dry weight. Sclerotia of *Botrytis* spp. were picked off the medium, cleaned, dried at 80° C. for 24 hours, and weighed. Those of *S. rolfsii* were brushed off the mycelia with a moist paint-brush, dried and weighed. A distinction was made between *initiation* of sclerotia (i.e. formation of the first visible sclerotia initials), *development* (i.e. growth to full size), and *maturation* (i.e. the development of the characteristic brown or black pigment). Whenever possible, the times of initial formation and the time taken to mature were recorded. The sclerotia of *S. gladioli* matured within 24 hours of initiation and thus separate data for the formation and maturation of these sclerotia are not given. Owing to the position of the sclerotia of *B. cinerea* and *B. allii* beneath the hyphal mat, these did not become apparent until they darkened, and in earlier experiments no attempt was made to record their initial appearance. In later work, it was found that the pale immature sclerotia were visible from the underside of the plate, provided that the media employed were not too opaque. Thus in some experiments, the time taken to form, as distinct from the time taken to mature, was recorded.

The influence of light was not a powerful factor in the production of sclerotia. There was slight inhibition of sclerotia of *B. cinerea* under continuous illumination, but this was probably due to slight differences in temperature. In general there was no distinction between the quantity of sclerotia produced in periodic light or in continuous darkness, though there might be slight differences in the rate of formation or maturation. Accordingly all cultures were incubated in the dark, unless otherwise stated.

The effect of temperature over the range 10° C.–37° C. was investigated. Although there was a distinct optimum for production of sclerotia by each of the six fungi, it was generally found that at any temperature which would support mycelial growth, sclerotial formation eventually ensued. Exceptions to this, however, were *B. cinerea* and *B. allii*, which at 25° C. produced vigorous hyphal growth and some conidia, but no sclerotia. These two fungi had the lowest optimum temperature for sclerotia among those studied. Paul (1929) and Peiris (1948) also found that the lowering of temperature increased the speed and amount of sclerotial formation by a number of *Botrytis* species. At the other extreme, the optimum temperature for the production by *S. rolfsii* was 30° C. Nakata (1927), who also worked on this fungus, claimed that the optimum temperature was 32° C.

In the experiments on nutrition the cultures were incubated at the temperature approximately optimal for the production of sclerotia for the particular species (i.e. *Botrytis* spp., 20° C.; *Sclerotinia gladioli*, 25° C.; *Sclerotium rolfsii*, 25–30° C.; *S. cepivorum*, 20–25° C.; *Rhizoctonia solani*, 20° C.) unless otherwise stated.

The effect of initial hydrogen-ion concentration of the medium was also determined. Glucose-peptone agar (glucose, 10 g.; peptone, 5 g.; KH_2PO_4, 1·75 g.; $MgSO_4$, 0·75 g.; agar, 20 g.; water, 1 litre) was adjusted to a range of pH 1·5–9·0 by suitable additions of tartaric acid or sodium carbonate and pH values were determined with a B.D.H. Comparator. Most of the species studied grew over a wide range of initial pH, but the production of sclerotia was favoured by an acid reaction. *R. solani*, however, showed an unusual tolerance of alkalinity and produced sclerotia on media with an initial pH of 8·8. Accordingly media used in nutrition experiments were adjusted where necessary to give an initial acid reaction. Changes in pH during growth were followed where it was thought that such changes might account for poor growth and scanty production of sclerotia.

TABLE I

The Effect of Various Sources of Carbon (at 1·0 per cent.) on the Production of Sclerotia by Six Species of Fungi

Source of carbon	Botrytis cinerea	Botrytis allii	Sclerotinia gladioli	Sclerotium rolfsii	Sclerotium cepivorum	Rhizoctonia solani
Glucose	++++ mmmm	++++ mmmm	++++ mmmm	++++ mmm	++++ mmmm	++++ mmmm
Fructose	++++ mmmm	++++ mmmm	++++ mmm	++++ mmm	+++ mmm	++++ mmmm
Sucrose	++++ mmmm	++++ mmmm	++++ mmmm	++++ mmm	++++ mmmm	++++ mmmm
Maltose	++++ mmmm	+++ mmmm	++++ mmmm	++++ mmm	+++ mm	++++ mmmm
Lactose	++++ mmm	+++ mmm	++++ mmm	+ m	+++ mm	+++ mm
Galactose	—	+++ mmm	+++ mmmm	—	+++ mm	—
Arabinose	+++ mm	—	—	+++ mmm	—	+++ mmmm
Starch	++++ mmm	++++ mmm	++ mmm	++++ mmm	++ m	++++ mmmm
Inulin	O.S. mm	+ mm	O.S. m	+ mm	+++ mmm	+ m
Mannitol	O.S. mm	O.S. mm	O.S. m	+ mm	O.S. m	O.S. m

— = not tested; m = poor slow growth; mm = poor growth; mmm = fairly good growth; mmmm = rapid, vigorous growth; + = very few sclerotia; ++ = few sclerotia; +++ = fairly numerous sclerotia; ++++ = very numerous sclerotia; O.S. = no sclerotia.

THE EFFECT OF THE NATURE AND CONCENTRATION OF THE SOURCE OF CARBON

Glucose, fructose, sucrose, maltose, lactose, galactose, arabinose, starch, inulin, or mannitol were added to a basic medium (KNO_3, 3·5 g.; KH_2PO_4, 1·75 g.; $MgSO_4$, 0·75 g.; agar, 20 g.; distilled water, 1 litre) to give concentrations of 0·5, 1·0, and 3·0 per cent. The experiment was performed in triplicate with each of the six fungi, with the exception that galactose and arabinose were not both used with the same fungus.

Table I summarizes some of the results of this experiment. It is clear that, in general, the greatest number of sclerotia developed on media which also favoured mycelial growth. With all species sclerotial production on glucose, fructose, sucrose, and maltose was good, and arabinose and galactose also proved to be suitable sources of carbon. Sclerotial production on lactose was good except with *S. rolfsii*. On starch, *B. cinerea*, *B. allii*, *R. solani*, and *S. rolfsii* grew well, and produced sclerotia in abundance, but *S. cepivorum* and *S. gladioli* both grew very slowly, and growth was sparse with only a few sclerotia. Growth on inulin was poor and sclerotia were few or absent except with *S. cepivorum* which grew well and produced fairly numerous sclerotia. On mannitol all the fungi grew poorly, and no sclerotia were produced by any fungus except *S. rolfsii*.

The generally poor effect of inulin and mannitol on both growth and sclerotial production could have been due to inability to utilize these substances as sole source of carbon. Those fungi unable to grow well on an inulin medium grew and produced sclerotia on such a medium in the presence of *Pyronema confluens* which is known to break down inulin. If 0·5 per cent. glucose were added to the inulin or mannitol media, growth and sclerotial production of all the fungi investigated were good, showing that neither inulin nor mannitol was harmful. Moreover, some of the species produced more sclerotia on an inulin or mannitol medium supplemented with glucose than on glucose alone. Therefore the unfavourable effects of inulin and mannitol are most likely due to failure to break these down rapidly enough to permit the development of a vigorous mycelium and of sclerotia, but where such a development was made possible by the presence of glucose some use was also made of inulin or mannitol. Where breakdown of inulin was brought about by *P. confluens* the products could be utilized by the sclerotia-producing species. Repeated subculturing of *B. cinerea* and *R. solani* on an inulin medium did not induce improved growth on such a medium, but *R. solani* showed improved growth on a mannitol medium after repeated subculturing on that medium, suggesting that an adaptive enzyme may have developed, or that a strain of the fungus able to use mannitol was selected by this method. This point was not investigated further.

When a particular species was provided with a carbon compound that it could readily break down and assimilate, an increase in the concentration of that compound, within the limits of the experiment, resulted in more vigorous mycelial growth and the production of a greater total number of sclerotia. With high concentration (3·0 per cent.) of carbohydrates, however, the time from inoculation to the initiation of sclerotia tended to increase and, moreover, the sclerotia, though numerous, matured slowly or not at all. This was particularly noticeable with *S. rolfsii*, sclerotia of which formed most rapidly at the lowest concentrations of suitable carbohydrates and also matured first on such media. Sclerotia of *R. solani* also matured most rapidly at low concentrations but formed first on media with high concentrations of carbon compounds.

In a series of experiments with *R. solani* and *S. rolfsii*, mycelial growth (measured as dry weight) and production of sclerotia was followed in liquid media with concentrations of glucose or sucrose from 0·1 to 5·0 per cent. The amount of sugar remaining in the media was estimated at suitable intervals by Somoygi's (1945) micro-method.

In an experiment with *S. rolfsii* the dry weights of the mycelial mats, including sclerotia, and of the sclerotia alone were estimated 6 weeks after inoculation (Table II) by which time the sugars had been used up.

The dry weights of mycelia and sclerotia were higher in the sucrose cultures than in the corresponding glucose ones. This difference was too great to be due to the higher carbon content of sucrose. (Sucrose ≡ 100/95 hexose, hence 5·0 ≡ 5·25 glucose, so that an increase of 5 per cent. in dry weight of mycelium on sucrose would not have been remarkable.)

TABLE II

Dry Weight (in mg. per 30 ml. medium) of Mycelial Mats including Sclerotia and of Sclerotia alone of S. rolfsii *on Media Containing different Amounts of Glucose or Sucrose*

Percentage sugar	Glucose		Sucrose	
	Mycelia + sclerotia	Sclerotia	Mycelia + sclerotia	Sclerotia
5·0	1,107	299	1,229	373
2·5	1,179	173	1,180	302
1·0	380	123	511	250
0·5	258	89	337	140
0·1	64	44	85	67

The greatest weights of mycelial growth were found at the highest concentrations of either sugar. The largest amounts of sclerotia were also produced at high levels of sugar but the ratio of dry weight of sclerotia to total dry weight of colonies was greatest at the lowest concentrations of sugars. Thus on 0·1 per cent. glucose and sucrose, the sclerotia represented 68·7 and 78·8 per cent. of the total weight of the cultures, whereas on 5·0 per cent. glucose and sucrose the proportions of sclerotia to the total weights were 28·2 and 30·4 per cent. respectively.

In a similar experiment with *R. solani* it was not possible to make a separate estimation of weight of sclerotia since these could not be readily separated from the mycelium, but visual inspection suggested that the effect of sugar concentration on total number and maturation of sclerotia was similar to the effect on *S. rolfsii*. Initiation of sclerotia of *R. solani*, however, took place earliest on relatively high concentrations of sugar in contrast to the results with *S. rolfsii*. Dry weights of cultures of *R. solani* on glucose and sucrose media showed less difference than those of *S. rolfsii*.

In a further experiment with *R. solani* the dry weights of mycelium, including sclerotia, and the amounts of sugar remaining in the media were estimated at approximately weekly intervals (Table III).

Rate of removal of sugar. It is clear from Table III that sucrose is readily inverted by this fungus, but that, while at the lowest concentration sucrose is used more rapidly than glucose, at high concentrations glucose is used more rapidly.

Rate and amount of growth. Growth is slightly more rapid on low concentrations of sucrose than on similar ones of glucose, thus showing a correlation with the rate of removal of sugar from the media. With high concentrations growth is again more rapid on sucrose than on glucose but reaches a maximum greater than the maximum on glucose, at about 24 days, after which the dry weight declines, presumably as a result of autolysis, so that the final dry weight is less than that on glucose.

Production of sclerotia. With both sugars sclerotial initials were formed earliest on media of high carbohydrate content, but maturation was most rapid at low concentrations. Table III shows that maturation did not occur on any medium until the sugar concentration had been greatly decreased. Nevertheless, maturation took place on media with high initial content of sugar while a considerable amount of residual sugar was present. Complete exhaustion of the sugar is therefore not essential, and maturation may depend rather on a reduction in hyphal growth-rate. Such a reduction occurs as a result of sugar exhaustion on media with a low initial concentration, but is probably induced by other factors, such as exhaustion of nutrients other than sugar, or the accumulation of inhibiting staling products, on high sugar media before the sugar is entirely used up. Table III shows a fairly close

TABLE III

Growth, Maturation of Sclerotia and Utilization of Sugar by R. solani *on Media Containing Various Amounts of Glucose and Sucrose*

Sugar	Days from inoculation	Initial sugar percentage in medium									
		0·1		0·5		1·0		2·5		5·0	
		Dry wt. of mycelium	Sugar used	Dry wt. of mycelium	Sugar used	Dry wt. of mycelium	Sugar used	Dry wt. of mycelium	Sugar used	Dry wt. of mycelium	Sugar used
Glucose	10	37	23	63	30	100	82	106	150	122	297
	17	43m	29	105m	148	210m	298	384	684	265	493
	24	45	29	90	149	235	299	420m	729	675	914
	31	50	30	95	149	193	299	420	746	805m	1,232
	38	55	30	111	149	205	299	470	746	868	1,305
	48	68	30	101	150	200	299	415	746	925	1,489
Sucrose	10	49	27 (0·1)	68	13 (135·6)	98	34 (259)	152	110 (329)	313	204 (370)
	17	45m	30 (0·0)	117m	143 (0·7)	233m	273 (10·9)	300	273 (489)	480	234 (1,274)
	24	48	30 (0·0)	105	148 (0·4)	207	293 (0·5)	520m	681 (65·7)	1,050	387 (1,127)
	31	50	30 (0·0)	119	146 (0·25)	216	291 (1·5)	450	691 (49·3)	751m	855 (617)
	38	51	30 (0·0)	125	149 (0·25)	224	292 (0·7)	447	734 (9·5)	796	976 (411)
	48	60	30 (0·0)	117	150 (0·0)	215	297 (0·5)	475	745 (3·7)	745	1,076 (411)
Total initial sugar			30		150		300		750		1,500

Dry weight of mycelium and total weight of sugar removed from the medium are both given in mg. per flask (which contained 30 ml. medium). m indicates time at which majority of sclerotia were mature. Figures in parenthesis in sucrose section of Table represent reducing sugar in medium in mg.

correlation between such a reduction in rate of vegetative growth and maturation of sclerotia, particularly with high concentrations of sugar.

Hawker (1939) showed that the contrasted effects of high concentrations of sucrose and glucose on growth and the production of perithecia of *Sordaria* (*Melanospora*) *destruens* could be partly, but not entirely, explained by the rate of inversion of sucrose by that fungus, which was such that a favourable concentration of reducing sugars was maintained over a relatively long period.

Rhizoctonia solani inverts sucrose more rapidly than does *S. destruens*, but even so the rate of inversion is such that, with high concentrations of sucrose, only part of the sugar is available in the form of hexoses at any time. Accordingly an experiment, similar to that of Hawker, was set up in which the effects of initial concentrations of 0·5 per cent. glucose and 5·0 per cent. glucose or sucrose were compared with that of a medium in which the initial concentration of 0·5 per cent. glucose was maintained by addition of that sugar at intervals during the experiment.

The results given in Table IV confirm those of the previous experiment but sampling was not continued long enough to show the fall in dry weight on the 5·0 per cent. sucrose medium. It is clear that the maintenance of a level of approximately 0·5 per cent. glucose by the addition of successive increments of this sugar gave results intermediate between those on a 0·5 per cent. glucose medium without subsequent addition and those on a 5·0 per cent. sucrose medium. Thus total dry weight of mycelium produced was less than that with a high concentration of sucrose but the total number of sclerotia was increased and maturation was delayed as on that medium or on one with a high initial concentration of glucose. After 19 days the amount of reducing sugar in the supplemented medium was greater than that remaining in the media with an initial concentration of 5·0 per cent. glucose or sucrose and whereas, in these media, rate of growth was falling off, in the supplemented one it was still increasing. This is reflected in the fact that in the latter the sclerotia were still colourless, but in the high sugar media they were beginning to colour, indicating that maturation had begun.

It has already been pointed out (p. 158) that maturation of sclerotia is associated with retardation of mycelial growth and that these may both take place before complete exhaustion of sugar in media with an initially high sugar concentration. Further experiments were designed to determine whether insufficient supply of certain growth-substances or exhaustion of nitrogen caused the retardation of growth on such a medium.

THE EFFECT OF VITAMIN B_1 ON SCLEROTIA PRODUCTION BY *S. ROLFSII*

All the fungi studied, with the exception of *S. rolfsii*, grew and produced sclerotia on suitable synthetic media without any additions of growth-substances. The production of mature sclerotia of *S. rolfsii* was increased by addition of aneurin (vitamin B_1) to certain media. Since aneurin is an essential component of co-carboxylase and since Hawker (1939*a*, 1942, 1944) has shown the importance of its effects on glucose utilization and respiration of *Sordaria* *destruens* and some other fungi, its effect on production of sclerotia by *S. rolfsii* was examined.

TABLE IV

Utilization of Glucose and Sucrose by R. solani

Sugar in medium	Days after inoculation	Residual sugar as per cent.	Weight of sugar (mg.) consumed per 100 ml. medium	Dry weight mycelium (mg.) per 100 ml. medium	Development of sclerotia
0·5% glucose	4	0·49	10	—	none
	6	0·419	81	—	i
	7	0·339	161	146	i
	10	0·016	484	270	i
	12	0·008	492	272	i
	14	none	500	230	sm
	17	,,	,,	214	sm
	19	,,	,,	190	sm
	21	,,	,,	200	sm
0·5% glucose made up to about original level at each sampling	7	0·342 (0·2)*	158	140	i
	10	0·235 (0·2)	665	450	ii
	12	0·356 (0·15)	744	636	ii
	14	0·277 (0·2)	1,223	960	ii
	17	0·34 (0·2)	1,960	1,330	ii
	19	0·20 (0·3)	2,300	1,552	ii
	21	0·279 (0·15)	2,621	1,840	si
5·0% glucose	4	4·904	96	—	i
	6	4·898	102	—	ii
	7	4·849	151	276	iii
	10	2·931	2,069	1,200	iii
	12	2·261	2,739	1,420	iii
	14	1·452	3,548	1,876	iii
	17	0·301	4,699	2,640	iii
	19	0·079	4,921	2·884	sim
	21	0·034	4,966	2,968	sim
5·0% sucrose	4	(a)0·049 (b)4·7 (c)4·747	253	—	iii
	6	(a)0·225 (b)4·22 (c)4·415	585	good growth	iii
	7	(a)0·247 (b)4·00 (c)4·236	764	540	iii
	10	(a)2·823 (b)1·328 (c)4·008	992	760	iii
	12	(a)3·674 (b)0·268 (c)3·766	1,234	1,080	iii
	14	(a)1·923 (b)none (c)1·827	3,173	1,664	iii
	17	(a)0·812 (b) ,, (c)0·761	4,239	1,950	iii
	19	(a)0·219 (b) ,, (c)0·208	4,792	3,019	sim
	21	(a)0·144 (b) ,, (c)0·136	4,864	3,160	sim

* Figures in parenthesis are percentage of glucose added at each sampling.

KEY: — = negligible growth; i = a few sclerotial initials; ii = rather more initials; iii = numerous initials; si = sclerotia immature, but full sized; sim = sclerotia beginning to mature; sm = sclerotia all mature.

(a) = free reducing sugars; (b) = sucrose; (c) = total of (a) and (b) expressed as sucrose.

S. rolfsii was grown on agar media in which the concentration of glucose was varied from 0·1 to 12·5 per cent. and that of aneurin added to the medium from 0·0 to 10 μg. per 100 c.c.s. medium. No attempt was made to estimate the amount of the vitamin present in the agar powder or carried over with the inoculum.

On the media without additional aneurin, sclerotial initials were formed 2 to 3 days later than on similar media supplemented with the vitamin. The concentration of aneurin had no effect on the number of sclerotial initials produced at any given concentration of glucose but the number maturing increased markedly with increase in aneurin (Table V).

TABLE V

Effect of Concentration of Glucose and Aneurin on Maturity of Sclerotia of S. rolfsii

Percentage glucose	μg. aneurin added per 100 ml. medium				
	0	1	2	4	10
0·1	M	M	M	M	M
0·2	90% M	M	M	M	M
0·5	5% M	90% M	M	M	M
1·0	I	90% M	M	M	M
2·0	I	90% M	M	M	M
3·0	I	50% M	90% M	90% M	M
5·0	I	50% M	70% M	80% M	90% M
7·5	I	50% M	50% M	70% M	80% M
10·0	I	I	25% M	30% M	50% M
12·5	I	I	I	10% M	25% M

M = all mature; I = all immature; percentages are approximate estimates, by visual inspection, of proportion of mature sclerotia.

A similar experiment on liquid media gave confirmatory results except that growth was negligible and no sclerotia were produced when no aneurin was added, suggesting that a certain amount was present in the agar used in the previous experiment.

Analysis of the effect of concentration of aneurin on rate of removal of sugar from the medium showed no significant difference at the range of concentrations studied.

From these experiments it is clear that mycelial growth, the production of sclerotial initials, and the maturation of the sclerotia all depend upon the presence of an external supply of aneurin. The requirements for mycelial growth are obviously very low and are satisfied by the traces present as impurities in the agar. It is not clear whether aneurin concentration has a direct effect on the *initiation* of sclerotia or influences it only through its effect on mycelial growth. In the experiment with agar media, however, initiation was delayed by low aneurin concentration, suggesting that there is a direct effect on sclerotial production in addition to the indirect effect through increase in mycelium. *Maturation* of sclerotia is clearly influenced by aneurin concentration and the requirements are much greater than those of initiation. With this fungus an increase in aneurin content of the medium to some extent reduces the retarding effect of high carbohydrate concentration on maturation of sclerotia.

THE CARBON-NITROGEN (C/N) RATIO

The inhibiting effect of high concentration of glucose and other carbohydrates referred to above (p. 158) might be due, in part at least, to the limiting effect of low nitrogen concentration. The nitrogen supply would be rapidly exhausted by the vigorous mycelial growth on media with high concentrations of carbohydrate and this might prevent maturation of sclerotia.

The importance of the ratio of carbon to nitrogen in the formation and maturation of sclerotia of *Botrytis allii* and *Rhizoctonia crocorum* has been pointed out by Peiris (1947) and Garrett (1949) respectively. Accordingly a series of experiments was undertaken to show the effect of the carbon-nitrogen (C/N) ratio on growth and the initiation and maturation of sclerotia of the six fungi under investigation.

Preliminary experiments showed that growth of all these fungi was good when the source of nitrogen was peptone, asparagine, or potassium nitrate in the presence of 2·0 per cent. glucose. Growth and sclerotial production were unsatisfactory when urea was the sole source of nitrogen, probably owing to excess ammonia production. The effect of ammonium salts as source of nitrogen varied according to the degree of acidity developing in the media as a result of growth. With ammonium tartrate and ammonium oxalate this was less than with ammonium sulphate or ammonium chloride, while ammonium nitrate was intermediate in effect. In general the two organic salts were better sources of nitrogen for the production of sclerotia than were the inorganic ones. Peptone, asparagine, or potassium nitrate were therefore used in the experiments on C/N ratios.

In the first series of experiments the fungi were grown on glucose-peptone media in which the percentage of glucose varied from 0·0 to 3·0 per cent. and that of peptone from 0·0 to 1·0 per cent.

Peiris (1947) showed that the number of sclerotia produced by a strain of *B. cinerea* on glucose-peptone agar increased with increase in glucose concentration while the number of conidia increased with increase in peptone concentration. He did not distinguish between initiation and maturation of sclerotia. The strain of *B. cinerea* used in the present investigation produced only a few conidia on any of the media used but sclerotia were produced freely on suitable media. As would be expected growth was poor and no sclerotia were produced on media lacking either carbon or nitrogen but the number of sclerotia increased with rise in concentration of either glucose or peptone in the presence of adequate amounts of the other (Table VI). With low concentration of peptone (0·1 per cent.) the number of sclerotia fell off and maturation was delayed, and finally inhibited, with increase in glucose concentration. Similarly with low concentrations of sugar (0·1 per cent.) increase in peptone concentration decreased the number of sclerotia. The highest level of peptone used (1·0 per cent.) gave only a slight increase of sclerotia even at the highest sugar concentration (3·0 per cent.). It is probable that a further increase in sugar at this level of peptone would give an increase in number of sclerotia.

The H-ion concentration of the media was determined at the end of the experiment and was within the range permitting production of sclerotia, so that the fall in number and delay in maturation of sclerotia on certain media was not due to the development of an unfavourable pH value. The inhibiting effects were thus associated with an excess of either carbon or nitrogen.

TABLE VI

Dry Weight (in mg.) of Sclerotia of B. cinerea *per Flask (30 ml. medium), at Different Levels of Glucose and Peptone*

Percentage peptone	Percentage glucose 0·1	0·5	1·0	3·0
0·1	84	294	380	27
0·3	71	315	546	1,031
0·6	70	411	713	1,469
1·0	16	410	736	1,485

Results with *B. allii* were similar in general trend but there was much less inhibition of sclerotia on media with extremely high or low carbon/nitrogen ratio than with *B. cinerea*.

With *S. gladioli* high concentrations of peptone markedly delayed and reduced the production of sclerotia, although this effect was counteracted to some extent by high glucose concentration. Maturation was delayed in media with a low C/N ratio. The inhibiting effect of high peptone concentration was correlated with the development of extremely alkaline conditions and such media were improved by the addition of tartaric acid. An interesting feature was the development, on media with high concentrations of glucose, of 'pseudosclerotia' which were softer and less sharply delimited than normal sclerotia. These might be interpreted as sclerotia which had failed to mature normally.

TABLE VII

Influence of Concentration of Glucose and Peptone on Initiation, Number, Size, and Maturation of Sclerotia of S. rolfsii

Percentage peptone	Percentage glucose 0·1		0·5		1·0		3·0	
0·0	6,	13	8,	15	8,	18	8,	20
	+	m	+	m	+	m	+	m
0·1	6,	13	6,	13	8,	13	9,	13
	++	m	+++	l	+++	l	+++	l
0·3	7,	15	7,	13	8,	15	9,	15
	++	m	+++	m	+++	l	++++	l
0·6	10,	20	7,	15	8,	15	9,	15
	++	s	+++	m	+++	m	++++	l
1·0	10,	22	8,	18	8,	16	9,	15
	++	v.s.	+++	s	+++	m	++++	l

In each section the left-hand figure = days to initiation of sclerotia, the right-hand one = days to maturation of sclerotia, + to ++++ represent arbitrary measures of number of sclerotia formed, l = large, m = medium-sized, s = small, v.s. = very small sclerotia.

Sclerotia of *S. cepivorum* were initiated first on media with high glucose concentration but a high C/N ratio retarded maturation. In contrast those of *S. rolfsii* were initiated first on media with low concentration of either carbon or nitrogen. Maturation on high concentrations of glucose was accelerated by increase in peptone and vice versa. The size of the individual sclerotia increased with increase in the C/N ratio (Table VII).

Sclerotial initials of *R. solani* were visible on all the media within 5 days of inoculation but the time taken to mature varied considerably. High concentration of either glucose or peptone combined with low concentrations of the other delayed maturation (Table VIII) but maturation was rapid when both substances were at a high level. Media with high concentration of glucose and low concentration of peptone became somewhat acid during the experiment but the pH value was within the range permitting the development of sclerotia.

TABLE VIII

Influence of Concentration of Glucose and Peptone on Number and Maturation of Sclerotia of R. solani

Percentage of peptone	Percentage of glucose							
	0·1		0·5		1·0		3·0	
0·1	8	+	10	++	34	+++	(−)	++++
0·3	8	+	8	++	8	+++	34	++++
0·6	13	++	8	+++	8	+++	8	++++
1·0	(−)	++	13	+++	8	+++	8	++++

The figures refer to days from inoculation to maturation of sclerotia, (−) = none matured at end of experiment (three weeks), + to ++++ represents arbitrary measure of number of sclerotia.

Thus with peptone as source of nitrogen all six fungi showed the same general trends: (i) the greatest number of sclerotia was initiated when both peptone and glucose were at relatively high concentration; the number tended to fall off when either substance was greatly in excess, (ii) maturation of sclerotia was delayed by high concentration of either glucose or peptone in the presence of inadequate supplies of the other, but the retarding effect of a high concentration of either substance was reduced by an increase in concentration of the other. In the single example of *S. gladioli* the position was complicated by the development of a toxic degree of alkalinity in all media with high concentration of peptone.

In a further series of experiments, which did not include *B. allii*, asparagine was substituted for peptone. In general the results showed the same trend as with peptone, but the inhibiting or retarding effects of high nitrogen concentration, particularly at low concentration of sugar, were usually more marked. The harmful effects of high concentration of asparagine were investigated in more detail with *B. cinerea* and *S. rolfsii*. The substitution of sucrose for glucose did not reduce the harmful effect of high concentration of asparagine on either fungus. The addition of growth-substances, in the form of a crude yeast extract or as pure aneurin, did not reduce this effect with

B. cinerea but did so with *S. rolfsii*. This last result was probably a result of the increased mycelial growth in the presence of aneurin (see p. 161) which led to a greater utilization of asparagine so that the development of a toxic degree of alkalinity was delayed. With *B. cinerea* the development of an alkaline reaction was delayed by the addition of tartaric acid to the medium and the harmful effect of high concentrations of asparagine was again reduced. It is thus probable that the effect of the carbon-nitrogen ratio was fundamentally similar, whether the source of nitrogen was peptone or asparagine, but that with the latter the rapid development of an alkaline reaction on media with initially high concentrations was a complicating factor.

A few experiments were also performed with *B. cinerea*, *S. gladioli*, *S. cepivorum*, and *R. solani* in which the source of nitrogen was potassium nitrate. In general the results were intermediate between those with peptone and asparagine. A full account of these experiments is given by Townsend (1952).

Discussion

The experiments described above show that nutritional requirements for the initiation of sclerotia differ from those for their further development and particularly from those for maturation, as judged by pigmentation. High concentration of carbohydrate and, to a lesser extent, of nitrogen tend to favour the initiation of large numbers of sclerotia but to retard or entirely inhibit their maturation. Media favouring mycelial growth, in general also favour the initiation of sclerotia but maturation of sclerotia does not necessarily follow. The *initiation* of sclerotia is dependent upon the amount of both carbon and nitrogen-containing nutrients available, but the amount permitting initiation is not necessarily sufficient for *maturation*. The latter process does not begin until mycelial growth is checked, either by exhaustion of nutrients or by some other cause. This might be due to the competition of vigorously growing hyphae for the available food-supply or it might be due to the necessity for a qualitative change in metabolism. Further work would be needed to decide this point.

When large numbers of initials are formed only a proportion of these mature. This may be due in part to physical overcrowding, but one would expect this to result in pigmented, but small, misshapen sclerotia rather than in failure to mature. Competition for food-supply is likely to prevent maturation of large numbers of initials and it might be possible to increase the number maturing by increasing the concentration of the medium above that of the experiments described in this paper. Garrett (1953) showed that competition for nutrient prevented the initiation of new rhizomorph initials in cultures of *Armillaria mellea* in which some older rhizomorphs were present. The possibility is not excluded that, both with sclerotia and rhizomorphs, mature ones may produce some substance which inhibits the initiation of others or the early development of those already formed. It is hoped that experiments in progress in this laboratory may help to distinguish between the factors favouring the initiation and early development of sclerotia and those inducing maturation.

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Studies in the Physiology of Parasitism

XXIII. On the Parasitic Vigour of Certain Bacteria in Relation to their Capacity to Secrete Pectolytic Enzymes

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With four Figures in the Text

ABSTRACT

1. Three organisms, *Flavobacterium* sp., *Pseudomonas* sp. designated No. 169 and *Pseudomonas syringae*, were compared with a pathogen *Bacterium aroideae*. Although they all produced pectolytic enzyme of equal activity on potato extracts, only the pathogen was able to parasitize the vegetable tissue, except when the water content of the latter was increased.

2. It was evident that success or failure of an attack was determined within 24 hours from inoculation. The events taking place during this period were studied in some detail with potato extracts and potato tubers. On dilute extracts the pathogen grew rapidly, with an acid drift of the medium, and pectolytic enzyme was detectable within a few hours from inoculation. The weaker organisms grew slowly, with no acid-forming tendency, and showed a delay in enzyme secretion. Differences in growth and in secretion of enzyme were further accentuated when the extract approached the strength of potato sap.

3. No obvious qualitative differences were found between the pectolytic enzymes secreted by the four organisms.

4. The failure of the weak organisms to attack normal potato tissue can be ascribed to their slower rates of growth and enzymic secretion which allow the host time to form a protective barrier. On the other hand, rapid growth and the secretion of enzyme within a few hours enabled *B. aroideae* to become established before a wound reaction could take place.

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A. INTRODUCTION

THAT isolates of certain soft-rotting bacteria secrete enzymes which disintegrate plant tissue has been known for at least half a century, as, for example, in the works of Potter (1902), Jones (1903, 1910), and van Hall (1903); and, as stressed by Brown (1955) in a review of the subject, the presence of such enzymes must be inferred wherever decomposition (maceration) of tissue is a feature of plant disease. There is, however, abundant evidence that, if an organism is able to secrete tissue-macerating enzymes into culture media and even into a decoction of a particular plant, parasitism by the organism on that plant does not necessarily take place. In other words, there is no general correlation between parasitic vigour and capacity to secrete tissue-macerating enzymes *in vitro*. A number of examples which illustrate this lack of correlation have recently been discussed by Brown (1955).

In an earlier number of this series, Gregg (1952) made a comparative study of four bacterial isolates, viz. *Bacterium aroideae*, *B. carotovorum* (i), *B. carotovorum* (ii), and *Phytomonas carotae*, with respect to pathogenicity and to activity in secreting a macerating enzyme system (protopectinase). When tested on normal potato tubers (and on three other common vegetables), these organisms were found to arrange themselves in a series of diminishing pathogenicity, ranging from the actively parasitic *B. aroideae*, through the two isolates of *B. carotovorum* which showed intermediate activity diminishing in the order as given above, and finally to the virtually non-pathogenic *P. carotae*. All four isolates gave pectolytic filtrates when grown in decoctions of the host plants, *B. aroideae* being in general most active in this respect. There was, in fact, a good correlation between pathogenicity and enzyme secretion for *B. aroideae* and the two strains of *B. carotovorum*. On the other hand, the very weakly parasitic *P. carotae* did not conform with this series, as its enzyme-secreting capacity was often little behind that of *B. aroideae*, the most active of the four isolates examined. The further analysis carried out by Gregg showed that the weakness of the *B. carotovorum* strains could be ascribed to an intrinsically slower rate of multiplication and to a pronounced tendency to develop a distinctly acid reaction in the medium during growth, a tendency which had the effect of slowing the rate of multiplication and of depressing enzymic activity. The latter effect arose in a twofold manner, from reduced secretion correlated with reduced growth of the organism and from the acid reaction which was unfavourable to the functioning of the enzyme (optimum pH, 9–10). The aberrant behaviour of *P. carotae* was left unexplained.

The aim of the present investigation was to attempt to carry the analysis farther and, as a first step to this end, a miscellaneous collection of isolates was examined in the hope of finding some which possessed the property of active secretion of pectolytic enzyme but which were non-pathogenic to the tissues of some of the common vegetables, in particular to potato tubers. A summarized account of this preliminary work will now be given.

B. SCREENING OF BACTERIAL ISOLATES AND PRELIMINARY OBSERVATIONS

Altogether about 40 isolates, obtained from many parts of Great Britain,[1] were examined in this connexion. Stock cultures of these were maintained on potato extract agar slopes (200 gm. potato per litre).

For inoculation purposes, 1-day-old cultures on yeastrel-peptone agar (yeastrel,[2] 3 gm.; bacteriological peptone 5 gm., to the litre) were used, this medium being chosen as giving better growth, especially with the Pseudomonads. Bacterial suspensions of standard turbidity, as determined by means of an EEL colorimeter, were pipetted into cavities, cut with a No. 2 corkborer after the method of Granger and Horne (1924), in tubers or tuber slices of potato which had been surface sterilized in the usual manner. An inoculum of 0·1 ml. suspension was applied to each. Incubation was for 3 days at 25° C.

TABLE I

Pathogenicity and Enzymic Activity of Bacterial Isolates

		Pathogenicity high		Pathogenicity low or absent	
Genus	No. of isolates	Enz. high (1)	Enz. low (2)	Enz. high (3)	Enz. low (4)
Bacterium	18	8	0	6	4
Bacillus	5	0	0	3	2
Pseudomonas	11	0	0	5	6
Unidentified	5	1	0	1	3
Totals	39	9	0	15	15

In the first general sorting-out experiment pathogenicity was assessed by a method of scoring. In later and more accurate tests the rotted tissue was rubbed out and weighed, or the volume of the cavity was determined after removal of rotted tissue, a correction being made for the volume of the original cavity.

For preparation of pectolytic enzymes, 20–25 ml. of potato decoction were placed in standard-sized medicine bottles and inoculated with 0·25 ml. of bacterial suspension. The inoculated bottles, laid on their sides so as to give maximum aeration, were incubated for 3 days at 25° C.

Pectolytic activity was measured by the disc method of Brown (1915) and later workers, the test being carried out in the presence of toluene in order to suppress further bacterial growth.

Table I gives the broad grouping of thirty-nine isolates in terms of pathogenicity and pectolytic enzyme secretion. Assessment of the former is based on the behaviour of inocula on whole tubers, this being the more rigorous test. Frequently it was found that, with weakly pathogenic isolates, there was negligible attack of whole tubers as against appreciable attack of slices, probably because the latter had received considerable washing with water, with consequent increase in water content, in the course of their preparation.

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[1] The writer is specially indebted to Dr. W. J. Dowson, Botany School, Cambridge, and to Mr. J. W. Egdell of the National Agricultural Advisory Service, South Western Province, for their great help in assembling the collection.

[2] Manufactured by Brewer's Supply Co., Ltd., Edinburgh.

It will be noted that there were no isolates which combined high pathogenicity with low enzyme production (col. 2). Those which fell into col. 4 were rejected as being of no interest for the present investigation. Chief interest attached to the members of col. 3 and especially to some of the Pseudomonads of that group which gave very active secretion of enzyme but no rotting of potato tubers.

TABLE II

Pathogenicity and Enzymic Activity of Four Isolates

Organism	Amount of rotting (gm.) in 3 days	Enzymic activity (R.T.)* after 3 days' culture on	
		Potato extract	Glucose-asparagin mineral salts medium
Bact. aroideae (Townsend) Stapp	0·96±0·2	22 min.	18 min.
Pseudomonas syringae van Hall	nil	22 min.	25 min.
Pseudomonas sp. No. 169	nil	12 min.	18 min.
Unidentified	nil	22 min.	8–24 hrs.

* R.T., reaction time, i.e. time for maceration of potato discs 0·5 mm. thick. Figures in Table are averages for 5 discs.

Sixteen isolates, taken from those in cols. 1 and 3 of Table I, were subjected to a more detailed test, with tenfold replication of the inoculations and with weighing of the amount of tissue (King Edward potato) attacked. On the basis of these determinations four isolates were finally selected as material for this study. The performance of these isolates is shown in Table II.

Some strains of *Pseudomonas syringae* have been reported (Jones and Dowson, 1950) as primary parasites of potato tubers in storage and it was hoped that a representative of that type could be included in the list studied. Unfortunately the pathogenic strains were no longer available and four strains of *Ps. syringae* obtained from Dr. Dowson proved to be useless for the writer's purpose, as their capacity to secrete pectolytic enzyme was slight and they were non-parasitic on potato tubers.

The organism labelled 'unidentified' in Table II, which had originally been isolated from glasshouse soil at Cheshunt, has been partially identified by its cultural reactions and has been classified as a species of *Flavobacterium.* Like the other three organisms it liquefied pectate gel. It was more active than the others in liquefying gelatine and much more active in hydrolysis of starch.

The four organisms of Table II were used throughout the course of this work. As only one of them was actively parasitic upon potato-tuber tissue, three other parasitic species, *Bacterium carotovorum*, *B. atrosepticum* and *B. phytophthorum*, were added to the list for particular experiments.

The pathogenicity of *B. aroideae* and the non-pathogenicity of the other three selected organisms was determined, in the first instance, with tubers of the potato variety King Edward as host tissue. Further experiments showed that the same relationship held for two other potato varieties, Majestic and Home Guard, and also for roots of turnip, carrot, and parsnip, and for buds of Brussels sprouts. Thus, whatever be the property which confers pathogenicity on *B. aroideae*, it is able to express itself over the range of environmental conditions represented by these various hosts.

When inoculated potato tubers were held at higher temperatures, 30° C. or 35° C. instead of the normal 25° C., the result was to increase the rate of attack of *B. aroideae* whereas that of the other three still remained very slight. Thus, within at least the limits tested, temperature does not tend to make the one group of organisms approach in activity to the other group. This effect, however, could be brought about by raising the water content of the tubers. Gregg (1952) has described such an effect for the four organisms, three parasitic and one non-parasitic, used in her work. Table III gives the results of an experiment with the four isolates of Table II, along the lines of Gregg's earlier work.

TABLE III

Effect of Water Content on Amount (gm.) of Rotting of Potato Cylinders

Organism	Treatment		
	Control	Soaked	Injected and soaked
Bacterium aroideae . . .	0.24	0·4	0·76
Pseudomonas syringae . . .	0·0	0·54	0·92
Pseudomonas sp. No. 169 . .	0·05	0·36	1·15
Flavobacterium sp. . . .	0·04	0·27	0·6

Average water contents: control, 79·1%; soaked, 83·1; injected and soaked, 83·3.

Cylinders of potato tissue (King Edward) were cut with a No. 10 cork-borer from surface sterilized tubers, rapidly washed in tap-water and cut into 1–1·5 in. lengths. They were then treated as follows:

(*a*) control
(*b*) soaked for 24 hours in sterile tap-water
(*c*) injected for ¼ hour under the air pump, then soaked for 24 hours.

The cylinders were inoculated in the manner already described and incubated for 24 hours at 25° C. The figures in Table III are the means of five replicates.

Increasing the water content of potato tubers by soaking or injection enabled the three weak isolates to attack potato tissue and to an extent comparable with the parasitic isolate. As was noted by Gregg, the effect of injection, over and above that of mere soaking, is out of all proportion to the very slight increase of water content produced by it.

The effect of moisture content in increasing attack was also brought out in some subsidiary experiments. A feature of *B. aroideae*, when placed on normal potato tissue, is that the region of the inoculum remains moist; with the weak organisms the inoculum 'dries up' within a few hours, the liquid presumably being absorbed by the sub-turgid tissue cells. This action can be prevented by adding water as necessary and such addition increases the attack of the weak parasites, e.g. from 0·04 to 0·16 gm. for *Flavobacterium* sp. The addition of fresh bacterial suspension is somewhat more effective and when the inoculation cavity is enlarged so that it can hold 1 ml. of inoculum the amount of attack becomes comparable with that shown by soaked and injected tissue.

On the other hand, with the volume of inoculum maintained constant (0·1 ml.), only a small or uncertain increase of attack was obtained by replacing the water of the bacterial suspension by such nutrients as potato extract, yeastrel peptone, 1 per cent. glucose or 1 per cent. asparagine.

Enzymic activity of the four selected isolates was determined in the first instance in extracts of King Edward potato (Table II). A parallel series of estimations was carried out with a variety of media and over a period of some months. The results can be summarized as follows:

1. All four organisms produced pectolytic enzymes of comparable activity on potato decoctions made from the three varieties King Edward, Majestic, and Home Guard. After 3 days' culture the medium showed an alkaline reaction in the neighbourhood of pH 7·5–8·5.
2. On turnip and carrot decoctions there was a less-marked drift towards alkalinity and in some the reaction was distinctly acidic, e.g. with the two Pseudomonads on turnip extract there was a drift from an original pH 5·3 to 3·9. Enzymic activity was associated with pH conditions, being less the greater the acidity. Addition of calcium carbonate to the nutrient prevented such acid development and led to an enzymic activity which was very similar for all the organisms and about equal to that on potato extracts.
3. High enzymic activity was shown on glucose-asparagine and yeastrel-peptone except for *Flavobacterium* sp. on glucose-asparagine (cf. Table II) which grows weakly on this medium. The lack of certain growth factors is thus indicated.

C. Initiation of Attack

It was abundantly clear that, when potato tissue was inoculated and incubated at 25° C., the results of inoculation were already manifest by the end of the first 24 hours; attack was either progressing or it had stopped, even if it had started at all. The difference in behaviour between aggressive and non-aggressive organisms was therefore to be sought within the first 24 hours of growth. The first problem was to determine whether any clear distinction could be drawn between the different types of organism in respect of rate of multiplication during the early phases of growth. Their relative capacity to produce enzyme at such an early stage was also of importance. These, and some other, effects will be dealt with in this section; consideration of defensive mechanisms will come up later.

i. *Relative growth-rates on potato extracts*

Growth of the organisms was estimated by changes in turbidity as recorded by an EEL nephelometer. Five ml. of clear sterile medium was pipetted into optically matched test-tubes. These were inoculated with 0·1 ml. of standardized bacterial suspension and incubated in a water bath at 25° C. The turbidity of the tubes was measured at half-hourly intervals, a 30 per cent. solution of the EEL turbidity standard being used for comparison.

Fig. 1 illustrates the growth of the four selected organisms in dilute potato medium (200 gm. potato to litre). Fig. 2 illustrates an experiment in which three other pathogenic bacteria, *Bacterium carotovorum*, *B. atrosepticum*, and *B. phytophthorum*, were also introduced. In the latter figure the curve for *Pseudomonas* sp. No. 169 is omitted as it followed closely the curves of the other two non-pathogenic isolates.

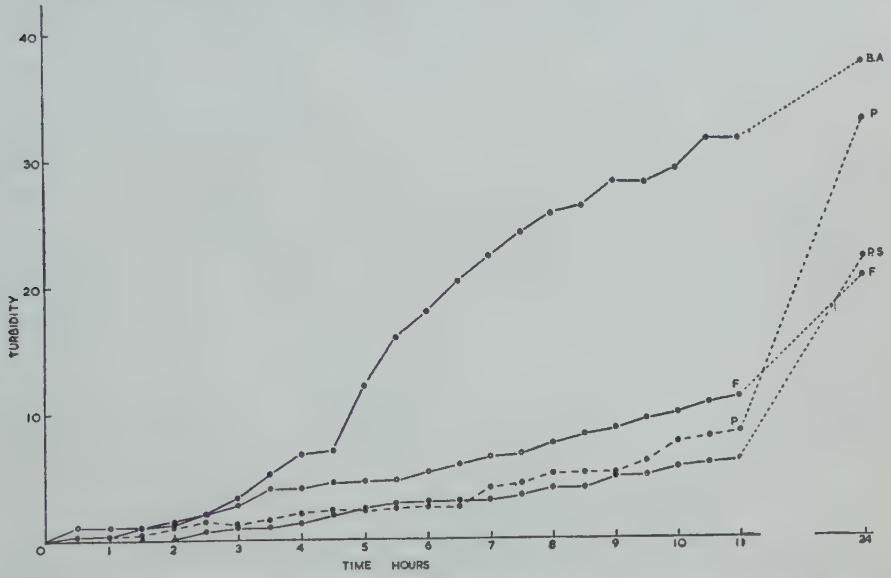

FIG. 1. Growth of *Bacterium aroideae* (B.A.), *Flavobacterium* sp. (F.), *Pseudomonas* sp. No. 169 (P.), and *Ps. syringae* (P.S.) on dilute potato medium.

Fig. 1 shows that the growth of *B. aroideae* becomes rapid after about 4 hours, at which time it begins to diverge strongly from the curves of the three other organisms. This divergence is still pronounced after 11 hours; after 24 hours, however, the curves have again approximated to each other, the rate of growth of *B. aroideae* having fallen off in the meanwhile. At this stage *Ps. syringae* and *Flavobacterium* sp. gave a turbidity similar to that of *B. aroideae* after 7 hours whereas *Pseudomonas* sp. No. 169 had almost reached the level of *B. aroideae*.

Fig. 2 brings out the same contrast, the curves of the four pathogenic organisms standing out clearly from those of the non-pathogenic.

The results shown in Figs. 1 and 2 were obtained with a dilute potato medium of arbitrarily chosen strength. When, however, a bacterial inoculum is put into plant tissue, the amount of water added is normally small in relation to the tissue of the host, so that the organism may soon find itself in a medium which approximates to the full-strength cell sap of the plant. It was obviously desirable, therefore, to study the growth-rates of the selected organisms in full-strength potato juice, in so far as such a preparation can be made.

Potato tubers were frozen for 24 hours, thawed, and the sap squeezed out. The latter was centrifuged to remove starch and passed under pressure through a Chamberland-Pasteur filter candle (porosity L7). Fig. 3 shows the growth of the four parasites and two of the weaker organisms over a period of 10·5 hours from inoculation. After 24 hours, the turbidity measurements for the organisms, in the order given in Fig. 3 from the top downwards, were: 104, 64, 49, 32, 10, and 1.

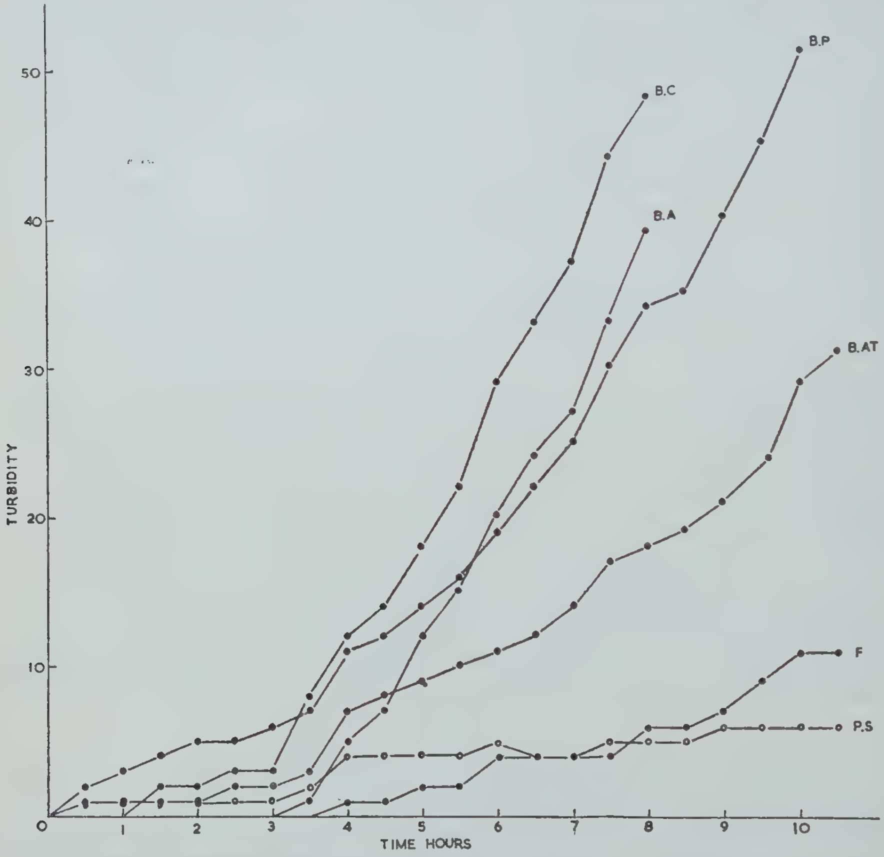

FIG. 2. Growth of *Bacterium aroideae* (B.A.), *B. carotovorum* (B.C.), *B. atrosepticum* (B.AT.), ***B.*** *phytophthorum* (B.P.), *Flavobacterium* sp. (F.), and *Pseudomonas syringae* (P.S.) on dilute potato medium.

Comparison of Figs. 1 and 2 with Fig. 3 shows that the effect of the more concentrated nutrient is to accentuate the initial growth-lag of all the organisms and more especially that of the non-pathogenic ones. In further experiments it was found that *Ps. syringae* made no appreciable growth after 24 hours on full-strength potato extract and that the early growth of all the organisms was delayed by the addition of 1 per cent. dextrose to dilute potato extract.

ii. *Enzymic secretion during early phases of growth*

Glaxo flasks, set up at 25° C. and each containing 250 ml. of dilute potato medium, were inoculated with 2·5 ml. of the various bacterial suspensions. This volume of medium forms a shallow layer at the bottom of the vessel.

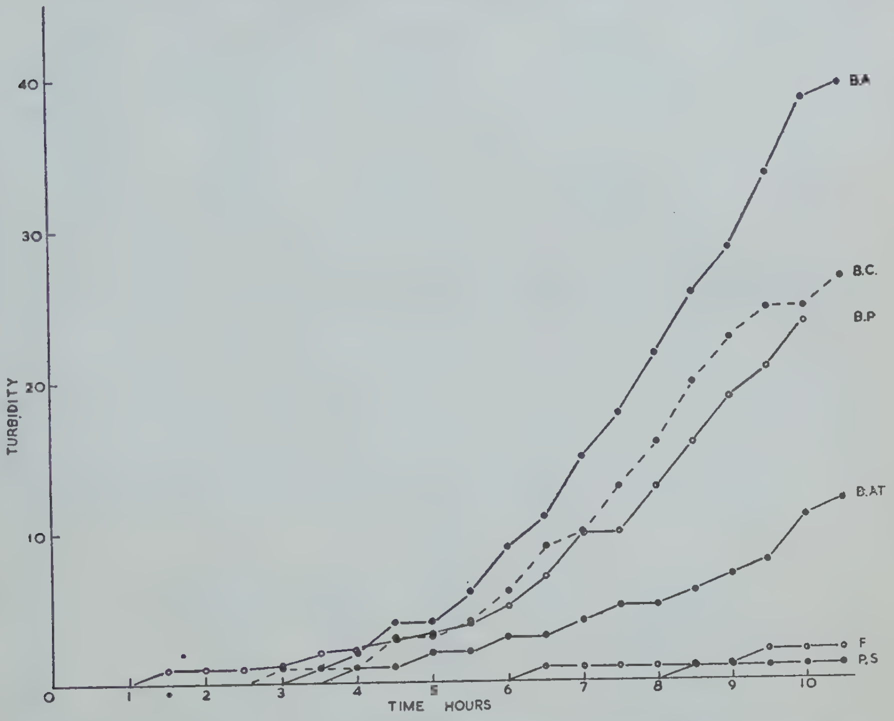

FIG. 3. Growth of *Bacterium aroideae* (B.A.), *B. carotovorum* (B.C.), *B. atrosepticum* (B.AT.), *B. phytophthorum* (B.P.), *Flavobacterium* sp. (F.), and *Pseudomonas syringae* (P.S.) on oxidized potato sap.

Samples were taken at 2-hourly intervals and, after pH adjustment to 7·5 with veronal buffer (Michaelis, 1931), enzymic activity was estimated by the tissue disc method. Table IV gives the result of a typical experiment, the data recorded being the times required (R.T.) for maceration of potato discs.

Table IV shows that there are marked differences in enzymic activity after 8 hours' culture but that after 24 hours they have largely disappeared. In the various experiments in this connexion, enzymic action by *B. aroideae* was always demonstrable after 4 hours' culture whereas the comparable stage for *Ps. syringae* was not reached until after about 10 hours. The other two organisms lay intermediately.

The corresponding series of enzymic determinations on full-strength potato juice was carried out only on a limited scale. This was because of the difficulty of preparing the requisite quantities of this medium, arising from the tendency of the filter candle to become choked. The only results available show the presence of enzyme in 24-hour cultures of the four parasitic organisms and of *Pseudomonas* sp. No. 169 but none in the remainder.

TABLE IV

Enzyme Secretion on a dilute Potato Medium

Incubation time (hrs.)	*B. aroideae*	*Ps. syringae*	*Ps.* sp. No. 169	*Flav.* sp.
2	No activity*	No activity	No activity	No activity
4	7–24 hrs.†	,,	,,	,,
6	4·5 hrs.	,,	,,	7–24 hrs.
8	2·3 hrs.	,,	7–24 hrs.	7–24 hrs.
24	40 mins.	35 mins.	30 mins.	45 mins.
48	30 mins.	25 mins.	25 mins.	35 mins.
120	20 mins.	25 mins.	—	—
144	—	—	25 mins.	35 mins.

* No action demonstrable after 24 hours.

† Maceration incomplete after 7 hours; discs devoid of coherence after 24 hours.

iii. *Time of onset of attack*

Experiments were carried out to determine when soft rotting of tissues began after inoculation. At half-hourly intervals two or three tubers were taken from inoculated batches and the surface of the inoculum cavity rubbed with the flat surface of a mounted needle to see if cells would come away.

TABLE V

Time of Onset of Attack on Potato Tubers at 25° C.

Organism	Time of attack (hrs.)
Bacterium aroideae	2–3
B. carotovorum	2–3
B. phytophthorum	2–3
B. atrosepticum	2–3
Pseudomonas syringae . . .	7–8
Pseudomonas sp. No. 169 . .	4–5
Flavobacterium sp. . . .	6–7

Each tuber carried two bacterial inoculations and one sterile water control. The onset of attack was taken as being the time when a distinction could be drawn between the inoculated and control cavities. The results obtained in a series of experiments were strikingly consistent and are summarized in Table V.

Incipient attack could be demonstrated in all cases, but much sooner with the four parasitic species than with the others.

The condition of the tissue, whether normal or soaked or injected, had no effect on the time at which rotting was detectable. There was, however, a marked effect on whether the attack developed further. With the last three organisms of Table V only the soaked, and more especially the injected, series gave appreciable soft rotting after 24 hours.

In the course of these experiments the presence or absence of free liquid in the inoculum cavity was recorded. It was clear that inoculations of *B. aroideae* showed a wet cavity at all times whereas those of the weaker isolates and the controls gradually lost the surface film of liquid. The average time required for absorption of the inoculum fluid by tubers of King Edward potato freshly taken from the clamp was 5–6 hours and this was shortened to 1·5–2 hours with tubers which had lain for 2 weeks on the laboratory bench, with resultant loss of water. Injection of the tissue with water strongly retards the uptake of the inoculum liquid but surprisingly does not prevent it altogether. It may be that the method of injection did not completely saturate all parts of the tissue or that vascular bundles in the neighbourhood of the inoculum actively transported the free liquid or it may simply be that the latter sank down through the intercellular spaces under the force of gravity.

iv. *pH drift and effect of pH upon growth*

In the experiments summarized in Table VI, pH changes were recorded at 2-hourly intervals during the early stages of growth but, as they were slight, only the later records are included in the Table.

TABLE VI

pH Drift on Dilute Potato Medium

Time	*B. aroideae*	*B. carot.*	*B. phytoph.*	*B. atrosep.*	*Ps. syringae*	*Ps.* sp. No. 169	*Flav.* sp.
Initial . .	6·4	6·2	6·4	6·4	6·45	5·8	6·1
8 hrs. . .	6·1	6·2	6·5	6·8	6·8	6·1	6·4
24 ,, . .	5·7	5·75	5·15	5·25	7·05	7·15	6·8
48 ,, . .	5·9	7·3	—	5·15	7·15	7·75	7·05

The outstanding point is that the four parasitic species showed an acid drift which had become reversed in the case of *B. carotovorum* after 48 hours. The three non-parasitic organisms did not show an acid drift and all finally gave a neutral to alkaline reaction. It may be added here that, of the collection of isolates originally examined (B, above) all but one (*B. salicis*) showed an alkaline reaction when grown for 3 days on potato extract.

It is not clear what significance, if any, is to be attached to this difference. The different behaviour may have been due to the slower growth-rate of the weak organisms in the early stages and they may in fact have shown a temporary acid drift between the 12th and 24th hours, when no observations were made. Even if fully established, such a difference cannot explain the important plant pathological difference already noted, viz. that the pathogenicity or non-pathogenicity of the organisms is already manifest within a few hours (at 25° C.) of inoculation, if, as seems likely, pH values in all the inocula have not yet moved appreciably from the starting-point.

Preliminary experiments with dilute unbuffered potato extract, set up at different initial pH's over the range 4–8, suggested that the optimal pH for growth of the two *Pseudomonas* species was on the acid side of neutrality.

With *B. aroideae* pH drifts were so pronounced as to prevent the drawing of any conclusion. If subsequently it should be thought that importance is to be attached to differential effects of pH upon bacterial growth in connexion with pathogenicity, the experiments will require to be carried out with adequately buffered media.

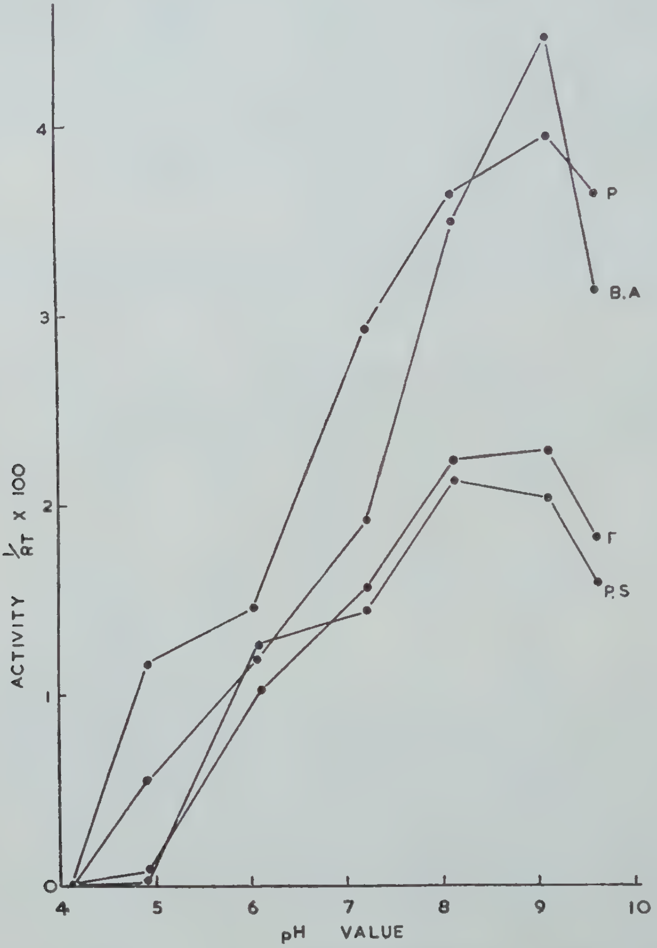

Fig. 4. Effect of pH value on activity of pectolytic enzymes of *Bacterium aroideae* (B.A.), *Flavobacterium* sp. (F.), *Pseudomonas* sp. No. 169 (P.), and *Ps. syringae* (P.S.).

D. Comparison of Pectolytic Enzymes

i. *Optimum pH for maceration*

The pH values of crude enzyme preparations were adjusted with decinormal NaOH or HCl and buffered with veronal acetate solutions (Michaelis). The pH was checked before and after the maceration of five potato discs. The mean maceration time was then calculated for each pH and the reciprocal (× 100) plotted as shown in Fig. 4. The optimum point for all preparations lies in the neighbourhood of pH 9 and, if there are minor differences, more detailed experiments would be required for their establishment.

ii. *Maceration and killing*

When pectolytic enzyme preparations of *B. aroideae* are allowed to macerate potato discs, the tissues gradually become limp and, if the discs are picked up with forceps at about the time when they have nearly lost all coherence, they bend round the instrument. The surface of the disc shows up matt, as against its original glistening appearance. Enzymic preparations of *Pseudomonas* sp. No. 169 behave in a similar way. *Pseudomonas syringae* and *Flavobacterium* sp., however, act differently. At the time when the cells have nearly lost their coherence, the discs still appear clear and glistening and can readily be picked up on forceps without much tendency to bend. They are brittle but not limp. If, at this stage, the discs are placed in hypertonic potassium nitrate containing neutral red dye, the majority of the cells become plasmolysed and the protoplasts are stained, evidence that these protoplasts are still alive. Similar treatment of discs macerated by preparations of *B. aroideae* and *Pseudomonas* sp. No. 169 shows most of the cells to be dead. This differential effect recalls the similar finding of Stevenson (1938) that such a difference was shown by enzymic preparations of *B. carotovorum* and *Botrytis cinerea*.

These observations merit a more detailed study as they represent the only marked differences yet recorded between the four crude enzyme preparations.

E. WOUND REACTIONS OF POTATO TISSUE

When a potato tuber is wounded, the first reaction to be seen is a suberization (browning) of the cell walls below the damaged surface (Priestley and Woffenden, 1922). This is followed by the organization of a meristem and the subsequent formation of a wound periderm. The subject has been reviewed by Bloch (1941, 1952). The formation of a wound barrier as a response to bacterial attack has been described by Appel (1906), Leach (1930), Jones and Dowson (1950), and many other workers.

The progress of the wound reaction, when potatoes were inoculated with the four selected bacteria, was traced on King Edward and Home Guard tubers. These were inoculated (Aug.–Oct.) in the usual way, samples removed after incubation for various periods at 25° C., and sections cut for investigation under the microscope. Certain staining techniques were tried but the main study was confined to unstained preparations. King Edward tubers were chiefly used for these observations; the sequence of events with Home Guard tubers was essentially the same but the responses throughout were somewhat more rapid.

Table VII summarizes the results obtained with King Edward tubers, when the cavities (made with a small cork-borer) were supplied with 0·1 ml. of water, potato sap or suspension of *Pseudomonas syringae*, alive or dead. Controls, with nothing added to the cavity, were also included.

The addition of water to the inoculum cavity (B) retards the normal wound reaction (A) by at least 24 hours, possibly because of local filling of intercellular spaces to the exclusion of air. On the other hand, addition of potato sap (C) more than restores the normal rate and intensity of suberization, an illustration perhaps of the Haberlandt (1902) effect. A similarly rapid and strong reaction was given by addition of suspension of live *Pseudomonas syringae* (E) in contrast to the reaction brought about by a suspension of dead organisms (D), which was not notably different from the effects of water.

TABLE VII

Progress of Wound-healing in Potato Tubers

Treatment of wound	24 hours	48 hours
A. Nothing added	Suberization (browning) of outer walls of first layer of undamaged cells lining cavity	As for 24 hours
B. Water added	No trace of suberization as yet: water absorbed	Sporadic occurrence of suberization but in outer walls only
C. Potato sap (oxidized) added	As for A, but several layers of cells affected; sap not yet absorbed	As for 24 hours, but extract now absorbed
D. Suspension of autoclaved *Pseudomonas syringae* added	Slight traces of suberization beginning	Localized patches of suberization in outer layer of cells
E. Suspension of living *Pseudomonas syringae* added	Marked suberization affecting several layers of cells	As for 24 hours

It was further shown that accelerated and intensified suberization caused by suspensions of live *Pseudomonas syringae* also occurred when suspensions of *Bacillus subtilis* or *Bacillus mesentericus* were similarly applied.

Development of a meristem, leading to periblem formation, took place 4–5 days after suberization.

Inoculations with *Bacterium aroideae*, the active pathogen, showed no sign of a wound reaction even after 3 days, by which time a considerable amount of rotted material was present. The suggestion therefore is that this organism kills the host cells before a wound reaction can take place. This is supported by the observation of Jones and Dowson (1950) that at ordinary temperatures *Bacterium carotovorum* invaded potato tissue without any trace of suberization showing, whereas, at a lower temperature or humidity, advance was slowed down and a suberized layer was produced by the host cells. On return to favourable conditions, the bacteria broke through this barrier and continued the rotting process; if, however, meristem formation had taken place, the bacteria were unable to break through.

The efficiency of the wound barrier in preventing attack by the four selected bacteria is illustrated by the following results. Tubers of variety King Edward were set up as usual, the cavities inoculated with water only and the tubers then incubated at 25° C. to allow wound healing to progress. At intervals they were inoculated with suspensions of the four selected bacteria. A delay of one day was found to be sufficient to suppress the small initial attack of which the three weak organisms were capable. On the other hand, 3–4 days' delay were necessary for prevention of attack by *B. aroideae*.

The relative speed of wound healing to that of attack by *B. aroideae* can be altered by change of temperature. According to Artschwager (1927) the optimal temperature for wound healing in potato is in the range 21–35° C.; at 5° C. healing is delayed by some 5–8 times. Lowering of temperature has a still greater effect on the growth and parasitic activity of *B. aroideae*; thus, according to Gregg (1952), attack on potato tubers was negligible at 10° C. and the writer's own work showed that growth of *B. aroideae* at 5° C. was very slow. Tubers inoculated with a suspension of *B. aroideae* and incubated at 5° C. showed only a trace of attack even after 7 days and by this time a complete suberized layer had been formed. At any time up to about the sixth day, transference of the tubers to 25° C. enabled the attack to take place; from about the seventh day onwards, wound healing had progressed so far as to prevent further invasion by this parasitic organism, even at the higher temperature.

The rate of initial attack by *B. aroideae* at 25° C. can be slowed down by reducing the number of organisms introduced with the inoculum, and if the number be reduced sufficiently, a stage is reached where attack fails, presumably because the wound healing process has been allowed sufficient time to become effective. Thus with the isolate of *B. aroideae* used in this work, and with suspensions containing approximately 10^8, 10^6, 10^4, and 10^2 organisms per 0·1 ml., the critical concentration for King Edward tubers was found to lie between 10^4 and 10^2.

F. DISCUSSION

The work described here is closely linked with the experiments of Fernando and Stevenson (1952), who analysed the respective behaviour of *B. carotovorum* and of *Botrytis cinerea* on potato tissue, with special relation to the properties of the pectolytic enzyme systems secreted. Over and above certain physico-chemical differences between the enzymes—e.g. distinctly different optimal pH points—an outstanding difference was that when enzyme preparations of the two organisms, which were equally active on testing by the disc method, were tested in small quantity on a massive piece of potato tissue, the *Botrytis* enzyme was much less effective than that of *B. carotovorum.* This difference was eliminated when the tissue was presented in the fully turgid condition, *Botrytis* enzyme now being as effective as the bacterial enzyme. Thus a sub-turgid condition (which very commonly prevails) of the host tissue confers resistance of the latter to *Botrytis* enzyme to a much greater degree than to the other. In this way one could understand the normal resistance of potato tubers to *Botrytis* attack and their susceptibility to *Bacterium carotovorum.*

The comparison of a fungal with a bacterial parasite, as in Fernando and

Stevenson's work, involves a complication arising from the different nature of the two organisms. The one is mycelial, and it may be able to penetrate and damage the host cells in a purely mechanical manner; the other can have no such power but it may be able to infiltrate into the intercellular spaces. The problem of analysis was simplified by selecting four bacteria, one of which was actively parasitic and the other three not so, and all four were about equally active in secreting pectolytic enzymes as tested on potato-extract medium after 3 days' and even after one day's growth.

Special attention was directed to the events taking place during the 24 hours following inoculation, as the success or failure of an attack is determined within this period. When a small inoculum (0·1 ml.) is placed on potato tissue it is rapidly absorbed, generally within 5–6 hours, by the tuber cells and within 24 hours a wound reaction is seen in the form of a suberization of the cell walls lining the damaged surface. These events take place if the weak bacteria are inoculated into the tuber. With the pathogen, however, although the inoculum liquid is gradually absorbed, the surrounding region remains moist and after 24 hours a rot can be seen penetrating several mm. into the tissue, and no wound reaction is detectable.

A critical study shows that, at 25° C., *B. aroideae* begins to rot the tissues within 3 hours from inoculation, i.e. before the inoculum liquid has been absorbed, but that the non-parasitic *Pseudomonas syringae* shows no effect until 8 hours after inoculation, by which time most of the inoculum liquid has disappeared.

The effects just described are paralleled by the behaviour of the organisms on potato decoctions. On dilute potato extract, pectolytic enzymes can be detected in the medium within 4 hours with *B. aroideae*, but only after 10 hours with *Ps. syringae*. Enzyme secretion by *B. aroideae* is demonstrable before much growth has occurred, and after a short lag period growth is very rapid. *Ps. syringae* grows slowly and the production of enzyme is correspondingly slow over the early stages. On full-strength sap the growth of *B. aroideae* is still rapid, but that of *Ps. syringae* is so reduced that little growth is detectable even after 24 hours. Thus the difference shown between the organisms on the dilute medium becomes accentuated on the stronger medium.

The sequence of events which follows the introduction of the inoculum may be visualized as follows. The organisms, at first in water, immediately find themselves in a dilute solution of cell sap which has come from the superficial cells of the inoculation cavity. As the liquid is absorbed by the sub-turgid adjacent living cells, the medium becomes more concentrated. By the time this has happened, an organism like *B. aroideae* has already produced enzyme in sufficient quantity to cause destruction of the neighbouring cells and this process continues so that decomposition of the tissue goes on indefinitely. With an organism like *Ps. syringae* the production of enzyme is slow, even under optimal conditions, and the removal of liquid from the inoculum further retards growth and enzymic secretion so that only negligible attack takes place. Meanwhile a defensive mechanism in the form of a suberization comes into play and any further action by the bacterium is rendered impossible. The effectiveness of this defence measure can be demonstrated even against *B. aroideae*, viz. when the inoculum of this organism is placed in the inoculation cavity 3 or 4 days after the latter has been made, so that the protective process has had time to go ahead before the pectolytic enzymes of *B. aroideae* are able to operate upon it.

So far there has been no detailed comparison of the enzyme systems of *B. aroideae* and the non-parasitic bacteria. This remains for further study. The only difference that has so far been detected is that *B. aroideae* enzyme appears to cause more rapid killing of living protoplasts than do the enzyme preparations of two at least of the other three organisms, but this effect requires further elucidation.

Finally, in the work described here, a host plant has been selected on which *B. aroideae* shows itself far more active than the other three organisms. It would be of considerable interest to compare and analyse the effects of *B. aroideae* and *Pseudomonas syringae* on the natural hosts of the latter, such as syringa, citrus, stock, and dwarf bean.

ACKNOWLEDGEMENTS

The writer wishes to record his thanks to Professor W. Brown, F.R.S., for suggesting the problem and for general guidance; to Dr. M. S. Lacey and Dr. R. K. S. Wood for their constructive criticism, and to the Agricultural Research Council for the Research Studentship which made the work possible.

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Nitrogen Fixation in Wild Legumes

BY

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With Plate IX

ABSTRACT

Isolations of the rhizobia from nodules of field plants of *Ulex europaeus* and *Medicago lupulina* have been examined for effectiveness in fixation of nitrogen by inoculating them into host plants growing in a medium free of combined nitrogen. The results indicate that vigorous fixation may be presumed to occur in association with field plants of the *Ulex* species, but that fixation in *Medicago* is probably decreased by the presence of ineffective rhizobia in some nodules. The general importance of fixation in wild legumes is discussed, and it is pointed out that due attention must be paid to non-legume genera with root nodules. These genera are locally abundant, and in former periods were still more prominent in some regions of the world.

INTRODUCTION

THE extent to which fixation of nitrogen is associated with wild legumes and thus to which such plants increase the nitrogen status of the soil has received little attention. By 'wild' is meant either a legume of no agricultural significance or one which, though used in agriculture, under the circumstances now being considered is growing in a natural community. On first consideration it may seem strange, in view of the undoubted nitrogen-fixing activities of legumes in cultivation, that any question should be raised as to the existence of a similar activity in legumes in the wild state. The suggestion has, however, been made (Wilson, 1940) that wild legumes are frequently associated with rhizobia of ineffective type, i.e. giving rise to nodules in which little or no fixation occurs. Thornton (1946) has reported on the frequency of such rhizobia in the nodules of clover growing essentially in a wild state in hill pastures in various parts of Britain, a finding confirmed for western Scotland by one of us (G. B.) in unpublished observations. It appears that certain soil conditions lead to a prevalence of ineffective rhizobia in the case of clover, and that the tendency for such conditions to develop is greater in the absence of cultivation.

On the other hand, circumstantial evidence of the frequent occurrence of active fixation in association with wild legumes is not wanting, as instanced by the ability of types such as gorse or broom to flourish in apparently barren soils. Campbell (1927) reported that in the southern states of the U.S.A. legumes figure very prominently among the plants recolonizing abandoned, exhausted cotton-fields and gravel wastes, and a more recent account (Whyte, Nilsson-Leissner, and Trumble, 1953) provides further examples of such an

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ecological succession under American conditions, which is consistent with the occurrence of fixation.

Given the presence of nodules on a wild legume, the extent of fixation turns largely on the question, referred to above, of rhizobial effectiveness. In the present work information on this aspect has been sought by a procedure frequently employed with cultivated legumes, namely, the preparation of isolations of the rhizobia from the nodules of field plants, followed by the testing of effectiveness by inoculating the rhizobia into host plants growing aseptically in a rooting medium free of combined nitrogen. The growth of the plants then provides an index of the effectiveness of the rhizobia in fixing nitrogen.

Two legumes native to Britain have been utilized, namely *Ulex europaeus* L. (Gorse or Whin) and *Medicago lupulina* L. (Black Medick or Yellow Trefoil). Gorse is also of widespread occurrence in central and western Europe, while black medick occurs over a large part of Europe, also in North Africa and western Asia. There are records of the cultivation of gorse in Britain as a source of winter fodder (Tansley, 1939), and some use continues to be made of black medick as a constituent of ley mixtures. According to cross-inoculation studies reported by Simon (1914) and Pieters (1927) the *Ulex* organism is most closely related to and is cross-inoculable with those of *Sarothamnus* and *Genista* so far as British legumes are concerned, while the *Medicago* organism is cross-inoculable with those of *Melilotus* and *Trigonella*.

METHODS

Totals of 78 isolations of the gorse nodule organism and 42 of that of black medick were made, each nodule employed being from a different plant. The gorse nodules were obtained from nine localities within a 25-mile radius of Glasgow, including roadsides, disused quarries, scrub-land, and situations near to high-tide level on the Firth of Clyde. The black medick nodules were obtained from a golf-course near Troon, Ayrshire, and from a roadside at Milngavie, Dunbartonshire. Considerable digging was required in finding the gorse nodules, and they could not be detected on all the plants investigated, though they were probably present. Black medick was heavily nodulated.

The nodules were surface-sterilized, then crushed in sterile water and the suspension streaked on to yeast-mannitol agar. About half of the gorse isolates were examined for the presence of *Bacterium radiobacter* (a frequent contaminant of rhizobial isolates) by the uric-acid method (Hofer, 1941), with negative results.

The intention was to use local seed, but such seed of gorse collected in autumn 1953 proved to have a heavy fungal contamination, while black medick proved to be insufficiently productive locally to yield the required amount of seed. Purchased seed, of British origin, was therefore used for both species. Gorse seed was surface-sterilized by immersion in concentrated sulphuric acid (Nutman, 1949), and black medick seed with absolute alcohol and mercuric chloride.

After germination in Petri-dishes containing a mineral-agar medium free of combined nitrogen, prepared according to the formula of Chen and Thornton (1940), the seedlings were transferred to test-tubes containing the same medium in slope form, those used for gorse being 20 × 3·5 cm. with 40 ml. of medium, those for black medick being 15 × 2 cm. with 10 ml. of medium. One plant was placed in each tube.

Inoculation was effected 3 days later by adding 0·5 ml. of a suspension of a nodule isolate to each plant tube, with five replicate tubes for each isolation. Uninoculated plants were also set up, some of them supplied with combined nitrogen in the form of ammonium sulphate. Growth was allowed to continue in the greenhouse for 4 months, after which individual dry weights (gorse) or fresh weights (medick) of the plants were determined. All the 170 uninoculated plants included in the trials remained free of nodules, showing that control over the incidence of the nodule organism was satisfactory.

In preliminary trials an isolation was selected which gave plants similar in vigour to those supplied with combined nitrogen, and in the actual tests this isolation was used as a standard against which the others were compared; this procedure facilitates the comparison of data obtained on different testing occasions. The effectiveness of each isolation was expressed as a percentage of that of the standard isolation, using the formula:

$$\text{Percentage effectiveness (P.E.)} = \frac{DWx - DWc}{DWs - DWc} \times 100$$

where DWx = weight of a plant associated with isolation x;
DWc = mean weight of non-nodulated control plants growing on the nitrogen-free medium;
DWs = mean weight of plants associated with the standard isolation.[1]

Since five plants were grown with each isolation, that number of separate estimates of the P.E. was obtained, permitting statistical treatment.

About half of the gorse isolates were tested in summer 1954 and the remainder a year later. The medick isolates were tested together in early spring 1955, the natural light being supplemented for the first 2 months by artificial light from a bank of fluorescent tubes.

DATA OBTAINED

Ulex europaeus

Nodules first appeared 2 weeks after inoculation, and all inoculated plants were nodulated within 4 weeks. The appearance of typical plants and root systems at the end of the growth period is shown in Plate IX, Figs. 1 and 2. Primary data, including total nitrogen in these particular cases, for typical plants from the 1954 tests are presented in Table I. They show that fixation of nitrogen in nodulated plants often approached 3 mg., and that the growth of such plants sometimes exceeded that of plants supplied with ample combined nitrogen.

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[1] The use of a standard isolation and of this formula was originally suggested to one of us (G. B.) by Dr. H. G. Thornton in another connexion.

TABLE I

Examples of Primary Data from the 1954 Gorse Tests

Type of plant*	Dry wt. of plant (mg.)	N content of plant (mg.)†	Percentage nitrogen
Non-nodulated	49	0·5	1·0
Nodulated, standard isolation	85	2·6	3·0
Nodulated, isolation No. 7	105	3·5	3·3
Nodulated, isolation No. 39	67	1·7	2·5
Nodulated, isolation No. 45	106	3·5	3·3
Non-nodulated, plus NH_4-nitrogen	88	3·3	3·7

* The values for non-nodulated and standard isolation plants are means. The remainder refer to individual plants.

† The mean nitrogen content per original seed = 0·3 mg.

The distribution of the mean P.E. values for the isolations tested is shown in Table II. There was frequently considerable variation in the growth of plants inoculated with a given isolation, factors contributing to this being the quicker establishment of some plants after transfer to the tubes, differences in seed size and genetical differences in the growth potentialities of the plants, and possible differences, again of genetical origin, in the facility with which individual plants can symbiose with a given isolation of the nodule organism, as demonstrated by Nutman (1946) for clover. These differences in growth were reflected in variations in the separate estimates of the P.E. for an isolation, so that statistical treatment was necessary. The analysis of variance on the P.E. values is summarized in Table III. As indicated there, two significant differences have been derived, on the basis of which the isolations have been arranged in three categories as in Table IV. It will be observed that no isolation falls into the ineffective class as here defined.

TABLE II

Distribution of Mean Percentage Effectiveness Values for Gorse Isolations

	Effectiveness values									
	0–20	21–40	41–60	61–80	81–100	101–20	121–40	141–60	161–80	181–200
Number of isolations	0	0	3	9	16	18	19	8	5	0

Medicago lupulina

Of the forty-two isolations tested, nine failed to produce nodules on any of the plants inoculated with them, while with some other isolations only a proportion of the plants nodulated. Purchase, Vincent, and Ward (1951) had a similar experience with other species of *Medicago*, and concluded that the infectiveness of some isolates was feeble and possibly affected by conditions. This is the most likely explanation of the present findings.

Among the plants that nodulated a very different position was revealed as compared with that in gorse, since a considerable proportion of isolations gave rise to plants differing little from the uninoculated control plants. Typical plants are shown in Plate IX, Fig. 3. The distribution of the mean P.E. values is indicated in Table V, but their interpretation is affected by the fact that variation between plants inoculated with a given isolation was greater than in gorse, so that the differences required for significance are larger. Statistical treatment similar to that employed for gorse showed that an isolation could not be regarded as significantly inferior to the standard isolation (P.E. 100) unless the P.E. was below 26, or not significantly superior to a completely

TABLE III

Summary of Analysis of Variance on the Percentage Effectiveness Values obtained in the Gorse Trials

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between isolations	645,669	86	7,508
Within isolations	483,317	336	1,438

Minimum differences required for significance ($P = 0{\cdot}05$)

(*a*) In comparing the mean P.E. of a given isolation with the standard isolation (5 and 15 separate estimates respectively):

$$\text{Difference required} = \sqrt{1438} \times t_{336} \times \sqrt{(\tfrac{1}{5}+\tfrac{1}{15})} = 39$$

(*b*) In comparing the mean P.E. of a given isolation with that of a completely ineffective strain which would have a P.E. near to zero and would yield plants similar to the non-nodulated control plants (5 and 10 separate estimates respectively):

$$\text{Difference required} = \sqrt{1438} \times t_{336} \times \sqrt{(\tfrac{1}{5}+\tfrac{1}{10})} = 41$$

TABLE IV

Classification of Gorse Isolations

Class	Definition in terms of mean percentage effectiveness	No. of isolations falling in class
Effective . . .	To be greater than 100−39 = 61	75
Ineffective . . .	To be less than 0+41 = 41	0
Intermediate . . .	To be in range 41 to 61	3

TABLE V

Distribution of Mean Percentage Effectiveness Values for Black Medick Isolations

	Effectiveness values					
	0–40	41–80	81–120	121–60	161–200	201–40
Number of isolations . .	16†	4	6	3	3	1

† Includes two isolations giving negative values.

ineffective isolation (P.E. 0) unless its P.E. exceeded 65. In these circumstances only a partial classification of the isolations is possible, as follows:

Effective isolations (P.E. > 65): number = 12.
Ineffective isolations (P.E. < 26): number = 14.
Unclassified isolations (the remainder): number = 7.

Thus more than half of the classifiable isolations were of ineffective type. In their origin these were not confined to either of the two field areas from which nodules were originally collected. A beaded type of nodulation, with close-set small nodules, was commonly shown with ineffective isolations (Plate IX, Fig. 4).

DISCUSSION

The above data show that in gorse only three out of the seventy-eight isolations that were examined failed to make possible a vigorous growth of test plants rooted in a medium free of combined nitrogen. These three isolations cannot be described as ineffective, since they enabled a growth of the host plant to occur which was significantly superior to that of plants without nodules. Thus for the particular area studied it may be concluded that ineffective forms of the gorse nodule organism, if present at all, are in very low proportion. In this respect gorse compares favourably with other legumes for which data are available. Thus Thornton (1946) provided data indicating that 21 per cent. of clover isolations obtained from varied British habitats were of ineffective type, while Umbreit (1944) found 25 per cent. of soya bean isolates from the Wisconsin area to be ineffective.

Though the nodulated gorse plant is thus equipped for vigorous fixation of nitrogen, the same is not true of black medick since more than half of the nodules examined yielded isolations of ineffective type (considering only those finally classified). It is of interest that Purchase, Vincent, and Ward (1951) found a majority of isolations from another species of *Medicago*, namely *M. laciniata*, to be likewise ineffective. Owing to lack of data for *M. lupulina* in cultivation it cannot be stated whether the proportion of ineffective rhizobia associated with such plants would be smaller than in the wild plants here examined.

While a similar examination of further types is desirable, on present evidence it may be concluded that some wild legumes fix nitrogen vigorously, but that others are handicapped by the presence of ineffective rhizobia.

It is generally recognized that in the agricultural regions of the world the importance of legumes in the maintenance of the nitrogen status of the soil has been greatly enhanced as compared with that which they have in natural communities of plants. Their actual importance in such communities is difficult to assess. Although the Leguminosae is a very large and widespread family, it is not ubiquitous; thus according to Whyte, Nilsson-Leissner, and Trumble (1953) it is but little represented in certain extensive grassland areas, including the pampas, the veld, and the steppes.[1] The same is true of some other plant communities, particularly on acid soils. Hutchinson (1944) concedes only that the evolution of the legumes 'presumably led to an increase in nitrogen fixation, which conceivably had significant biogeochemical results'. Caution is clearly necessary here since vigorous plant growth obviously occurred prior to the Mesozoic.

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[1] This statement is assumed to be authoritative, although it does not entirely agree with earlier accounts of the vegetation of these areas.

If it is accepted that fixation in legumes has been of some general importance to the plant kingdom, then the same conclusion must be extended to the nodule-bearing non-legume genera (see Bond, Fletcher, and Ferguson, 1954; Bond, MacConnell, and McCallum, 1956). It has been shown for most of these that they resemble legumes closely in their nitrogen-nutrition; they have the same ability to grow in nitrogen-poor media, and it cannot be doubted that they leave the soil enriched in nitrogen. These genera are still abundantly represented in some regions, as is illustrated, for example, by the dominance of *Myrica gale* over considerable areas of peat bog in Britain, and by the prominence of *Shepherdia canadensis* in sub-arctic regions of Canada (Raup, 1941). It seems likely that in pre-agricultural eras such genera were even more prominent in some regions. Tansley (1939) reviewed the evidence relating to the vegetation of Britain in post-glacial ages, and concluded that *Alnus glutinosa* was very prevalent, particularly in the Atlantic period (5,000–3,000 B.C.) when it appears that the better-drained part of the country was largely covered by mixed oak forest, including alder, and the lowland marshy regions by alder woods. Such extensive afforestation leaves little room for legumes. Fraser and Godwin (1955) and Walker (1955) refer to the prevalence of *Hippophaë rhamnoides* in many parts of Europe in Late-glacial periods, while in connexion with other regions the genus *Coriaria*, which also has root nodules, is of special interest. Good (1930, 1947) considers this to be at present the most widely scattered of all Angiospermous genera, since it occurs in Central and South America, the Mediterranean region, eastern Asia, and New Zealand, and from this and fossil evidence he concludes that the genus, though now in a senile condition, was in former times prominent in many parts of the world. It is certain that the past and present biogeochemical importance of non-legume nodule-bearing plants is worthy of further study.

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EXPLANATION OF PLATE IX

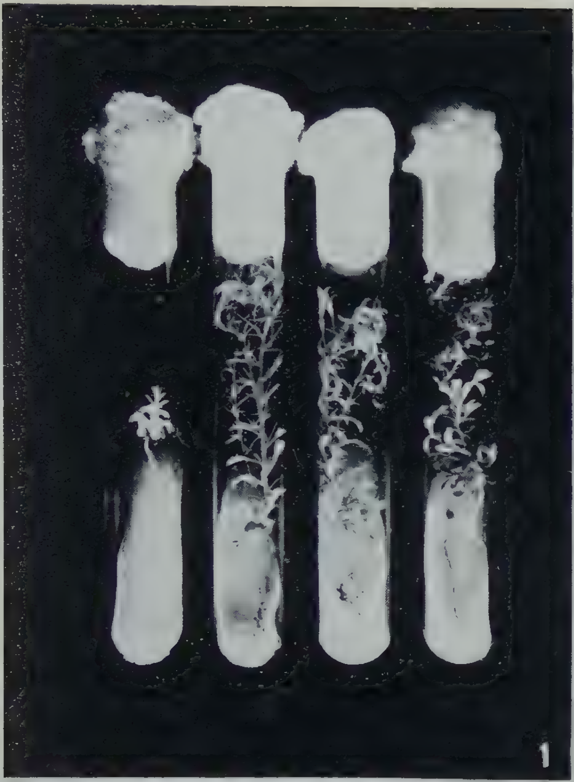
Illustrating the article by J. T. MacConnell and G. Bond on 'Nitrogen Fixation in Wild Legumes'.

Fig. 1. Typical plants of *Ulex europaeus*, including (left to right) a non-nodulated control plant, two nodulated plants inoculated with the standard isolation of the nodule organism, and a non-nodulated plant supplied with ammonium sulphate. (×$\frac{1}{3}$.)

Fig. 2. Nodulated root system of plant of *Ulex europaeus*. (×1.)

Fig. 3. Typical plants of *Medicago lupulina*, including (left to right) a plant nodulated by an effective isolation, a non-nodulated control plant, and a plant nodulated by an ineffective isolation. (×$\frac{2}{5}$.)

Fig. 4. Nodulated root systems of plants of *Medicago lupulina*: on the left the nodule organism concerned is of ineffective type and the nodules take the form of numerous bead-like swellings (seen more clearly with a hand-lens); on the right the organism is of effective type. (×2.)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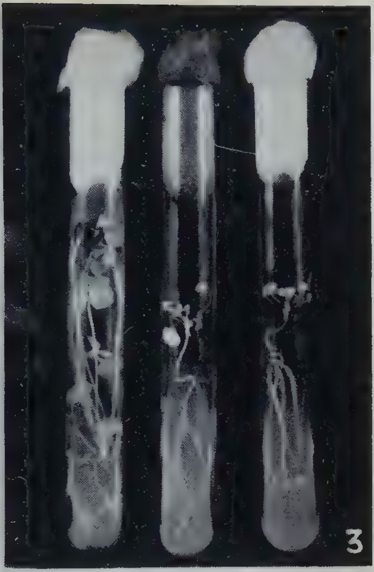
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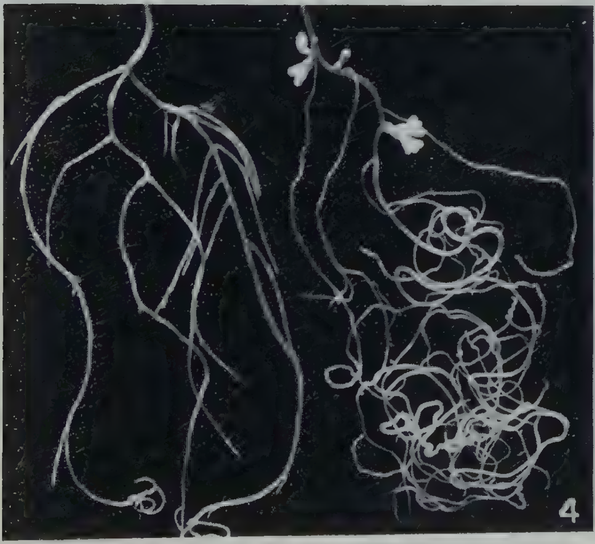
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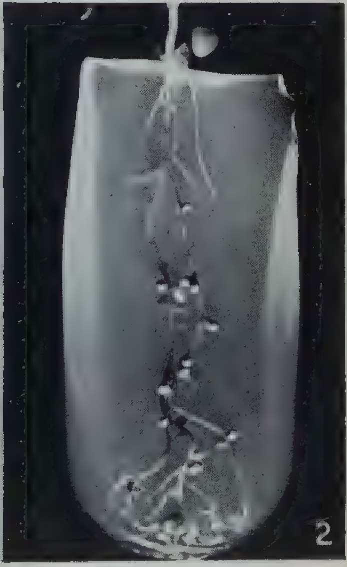
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J. T. MacCONNELL *and* G. BOND

Nitrogenous Compounds and Nitrogen Metabolism in the Liliaceae

III. Changes in the Soluble Nitrogen Compounds of the Tulip and their Relation to Flower Formation in the Bulb

BY

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ABSTRACT

The principal free amino-acid of the vegetative tulip bulb is arginine and the alcohol insoluble nitrogen fraction contains combined arginine to the extent of 47 per cent. of the nitrogen. In the green leaf the amides glutamine and asparagine occur whereas free arginine is virtually absent. These amides do not occur appreciably in the vegetative bulb.

By appropriate temperature treatments flowers were initiated and after the floral parts were formed they were caused to develop to the point of emergence from the bulb. The concomitant changes in the soluble nitrogen compounds were determined.

The dramatic change in the composition of the soluble nitrogen involving a shift from the predominance of arginine to a relative increase in glutamine and asparagine occurs when the already initiated flowers develop.

γ-methyleneglutamine and γ-methyleneglutamic acid which are prominent in the tulip seem not to be greatly affected by the treatments that cause floral initiation and development. Their metabolism seems superimposed upon the nitrogen metabolism based on arginine in the bulb and amides in the leaf which is responsive to the treatments involved in flower formation.

The occurrence of arginine in the tulip

SOMEWHAT early in the chromatographic examination of the nitrogen compounds found in *Tulipa gesneriana* (Zacharius, 1952; Zacharius, Pollard, and Steward, 1954) two features became obtrusive. These were, first, the conspicuous amount of free arginine in the bulb, second, the occurrence of the substances now known to be γ-methyleneglutamine and γ-methyleneglutamic acid. These observations prompted certain other experiments (Cathey, 1955) which give a first idea of the way in which these substances change during the course of development. Though incomplete the data permit the dominating role of arginine in the bulb, in contrast to the amides in the green leaf, to be stressed.

Floral initiation occurs in the tulip bulb and the optimum temperatures for (*a*) initiation of the floral parts, (*b*) their subsequent development have been well worked out by the Dutch workers (Blaauw *et al.*, 1925; Mulder and

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Blaauw, 1925; also see Beijer, 1952). Floral initiation occurs within the central scale of the bulb and, as the development of the bud proceeds, there is progressive utilization of successive scales by the growing flower bud. In this situation changes in the nitrogenous compounds concomitant with floral initiation and development may be traced.

The quantitative chromatographic methods used were substantially those described by Thompson, Zacharius, and Steward (1951) with such additional features as became necessary after the identity of γ-methyleneglutamine and γ-methyleneglutamic acid became known. (Instead of collidine-lutidine, butanol-acetic acid was used as the second solvent and this necessitated the use of the appropriate standard curves for all the substances determined.) All the soluble compounds were extracted with alcohol at a concentration of about 75 per cent. and the extracts were concentrated at room temperature prior to phenol: butanol-acetic acid chromatography on Whatman No. 1 papers. (Where large amounts of arginine occurred, special care was taken in this way to avoid confusion with glutamine.)

The nitrogenous composition of the vegetative bulb, in which floral initiation was at a minimum, can be seen by observations made on two types of material.

Bulbs from plants which had borne flowers were dug about June 1 and these were extracted and analysed immediately thereafter, while comparable bulbs were allowed to remain in dry storage until the following January when the floral development was completed.

For more precise observations on the stages of floral development, bulbs (var. William Pitt) were obtained by air from Puyallup, Washington, where they were dug on July 1. On receipt these bulbs showed no floral initiation, having formed only two to three leaves in the apex. After 2 weeks of storage at 31° F. the bulbs were transferred to 68° F., the optimum temperature for floral initiation, and there left for periods up to 26 days by which time floral initiation was completed. From eight bulbs at each period, samples were taken for microscopical examination of the dissected apices (one bulb) and for examination of the nitrogenous compounds during this period. After complete floral initiation (26 days) bulbs were transferred to 48° F. for one month in order to investigate the type of chemical changes which ensue while the flowers *developed* after they were initiated. During the latter period the floral organs reached an advanced stage of development and were ready to emerge from the bulb. The stages of floral initiation as observed on the dissected bulbs at the different stages when samples were taken are shown below:

0 days:	Growing points completely vegetative.
6 days:	Early stages of floral initiation—first whorl of petal primordia visible.
15 days:	Both whorls of the corolla clearly formed.
19 days:	Anthers completely differentiated. Carpels being formed.
23 days:	All floral parts present.
26 days:	Floral initiation completed.

The storage reserves of the vegetative bulbs

When freshly lifted from the soil and before the main events of floral development, the tulip bulb has a characteristic nitrogenous composition both with respect to the alcohol soluble and alcohol insoluble moieties. (Zacharius, 1952.) The protein of the bulb scales of the tulip, as lifted from the soil on June 1, proved to be unusually rich in arginine. The alcohol insoluble residue was acid hydrolysed and chromatographically analysed. Table 1 records the data as (*a*) the relative composition of the protein-N in terms of the percentage of the total alcohol insoluble nitrogen so determined which is represented by each amino acid and as (*b*) the nitrogen content of the bulb, as μg. per g. dry weight, which was represented by each amino acid in the insoluble fraction. The recovery of the alcohol-insoluble nitrogen by these determinations was good. Whereas the separate amino-acids, necessarily excluding histidine and tryptophan which were not determined, represented an alcohol-insoluble nitrogen content of 13·2 mg. per g. dry weight; direct Kjeldahl determination gave 15·7 mg. nitrogen per g. dry weight: thus 83·8 per cent. of the tulip protein-N was directly determined in this way. As Table I shows, 47·7 per cent. of the alcohol-insoluble nitrogen was accounted for as arginine. This unusually high content of arginine—apparently unique among bulk protein analyses (Steward and Thompson, 1954)—is comparable to that found in protamins.

TABLE 1

Quantitative Analysis by Paper Chromatography of the Bulk Proteins of Tulip Bulb Scales*

Amino-acid	Nitrogen of the amino-acid as % of alcohol insoluble nitrogen	μg. alcohol-insoluble nitrogen, in the form of each amino-acid, per g. dry wt.
Aspartic acid	8·04	1,060
Glutamic acid	5·71	752
Serine	4·35†	572
Glycine	9·39	1,240
Threonine	1·17†	154
Alanine	2·76	364
Lysine	7·79	1,030
Arginine	47·7	6,280
Proline	4·47	588
Valine	3·10	408
Leucine	4·70	618
Phenylalanine	0·12	16
Tyrosine	0·08	10
Cysteine	0·70	92
Methionine	0·06	8
Hydroxyproline	None	None
Total	100·14	13,192

* histidine and tryptophan not analysed.
† corrected for losses due to hydrolysis (Rees, 1946).

Changes in soluble nitrogen compounds during development

The preceding paper of this group has shown that amides, glutamine, asparagine, and γ-methyleneglutamine are prominent in the composition of the expanded green leaf. The familiar amides are either totally absent (asparagine), or present in the bulb only in small amounts (glutamine), whereas γ-methyleneglutamine occurs in relatively much larger amount. However, the conspicuous feature of the alcohol-soluble nitrogen fraction of the bulb is the most unusual quantity of free arginine which is present. By qualitative comparisons of material harvested on June 1 and after storage until January 23 the broad changes that occurred in the soluble nitrogen fraction could readily be seen. These are indicated in Table II which discriminates between the vegetative and floral parts. The conspicuous features noted were:

TABLE II

Ninhydrin Reacting Compounds of the Tulip Bulb

Amino compound	Fleshy† leaf bases and flower initial June 1	Fleshy† leaf bases of bulb Jan. 23	Yellow† leaves of bulb Jan. 23	Stamens† Jan. 23	Pistils† Jan. 23	Sepals† and petals Jan. 23	Mature† green leaves Jan. 23
Cysteic acid	—	tr	tr	*	tr	tr	—
Aspartic acid	****	***	***	***	***	***	**
Glutamic acid	***	****	****	***	**	***	***
Serine	*	***	**	***	***	***	**
Glycine	—	—	—	—	—	—	—
Asparagine	none?	**	**	***	**	**	none
Threonine	*	**	—	**	*	*	—
Alanine	**	****(G)	***	***	***	***	***
Glutamine	*	****(G)	****(G)	****(G)	****(G)	****(G)	**
Lysine	*	none?	none	**	***	**	—
Arginine	****(G)	****	****(G)	****(G)	****(G)	****(G)	—
Proline	—	***	**	***	***	***	—
Valine	**	****(G)	**	***	***	***	**
Methionine sulfone	—	tr	—	tr	tr	tr	—
Leucine(s)	*	****(G)	**	***	***	***	**
Phenylalanine	} tr	*	tr	tr	} *	} *	—
Tryptophan		**	tr	*			
Tyrosine	tr	*	*	*	*	*	—
γ-aminobutyric acid	tr	**	*	—	*	*	*
γ-methyleneglutamine	****	***	****(G)	**	***	**	****(G)
γ-methyleneglutamic acid	***	***	***	*	****	****	**

(Four other substances appeared on these chromatograms in small amount or sporadically) (G) denotes the compound(s) present in greatest amount.

† Extract treated with hydrogen peroxide. } Either—or

(*a*) Arginine remained the dominant constituent of the alcohol soluble nitrogen compounds of the bulb throughout its storage history and *until the expanded green leaf was formed.* In the green leaf free arginine was virtually absent, though it is the major constituent in sepals, petals, and the yellow leaves within the bulb as well as in the fleshy scales.

(*b*) Although glutamine was scanty in the fleshy scales of the bulb prior to floral initiation, it was present in large amount after the flowers had developed to the point of emergence and it then occurred in all the organs examined

(January 23). Although much less conspicuous, asparagine occurred in these samples also. The amide (glutamine+asparagine) content of the emerged green leaf, however, was notably less than that of the various organs in the bulbs.

(*c*) γ-methyleneglutamine was present throughout in the bulb but was most conspicuous in the yellow leaves before they had emerged and in the green leaves. Reference to Part I of this group of papers will show that the γ-methylene compounds are weakly represented in seeds in comparison with leaves.

(*d*) While aspartic and glutamic acids were present throughout the storage life of the bulb the main changes which ensued between the onset of floral initiation and the virtually complete development of the flower, prior to its emergence, is the reappearance in quantity of such amino-acids as alanine, the leucines, and valine especially in the scale leaves and in the still yellow, young, foliage leaves.

TABLE III

Time Course in Floral Development and Changes in Total Soluble Nitrogen of Tulip Bulbs

Period No.	Days at 68° F.	Days at 48° F.	Status of flowers	μg. sol. N. per g. fresh weight
0	0	—	Floral initials absent	518
1	6	—	First whorl of petals being formed	532
2	15	—	Second whorl of petals developed	417
3	19	—	First whorl of anthers present	501
4	23	—	Second whorl of anthers developed	314
5	26	—	Pistil formed	622
6	—	1 month	Flower fully mature and ready to emerge	730

(*e*) This suggests that somewhere between these two extremes, i.e. prior to floral initiation and after completion of the development of the flower, a remobilization of the soluble nitrogen compounds occur.

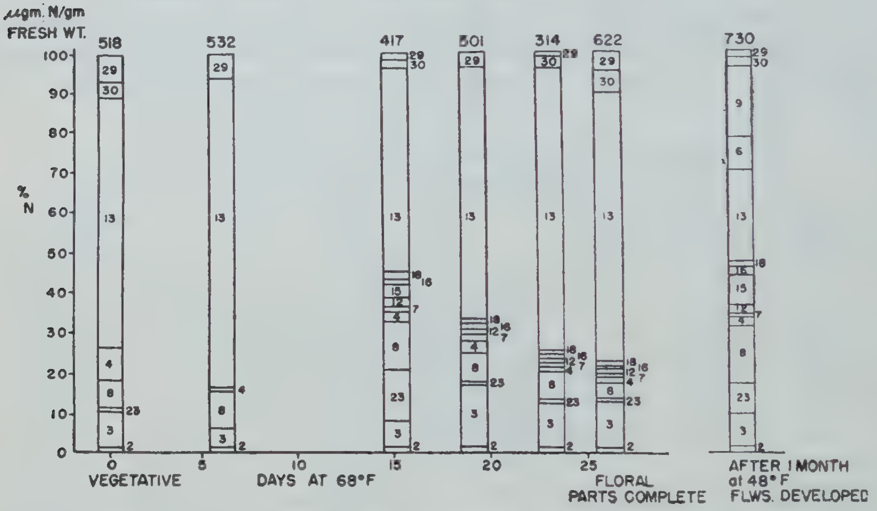
The development of the tulip flower from initiation to full development was traced by exposing bulbs of the variety William Pitt to 68° F., the temperature stated to be the optimum for initiation of the floral parts. Periodically bulbs were removed, the flowers dissected, and at critical stages bulbs removed for chromatographic analysis of the alcohol soluble nitrogen compounds. By summation of the nitrogen accounted for by individual amino acids an estimate can be made of the changes in the pool of total soluble nitrogen compounds. These data are shown in Table III.

These data show two stages at which the soluble nitrogen reserves of the bulb were much depleted, i.e. periods 2 and 4. These correspond to stages at which (*a*) the most rapid initiation of the corolla has occurred (Period 2) and (*b*) the androecium was fully initiated and that of the gynoecium had already commenced. The large reserves of basic soluble nitrogen, in the form of arginine, nourished this development but the pool of soluble nitrogen must have been replenished (presumably from the alcohol insoluble portion) for, when the floral organs were fully laid down (Period 5), or even ready to emerge (Period 6), there was actually more soluble nitrogen in the bulb than before floral initiation began. This replenishment must, therefore, occur from the arginine rich storage reserve of the alcohol insoluble nitrogen referred to above.

The figure shows the changes in the relative composition of the alcohol soluble-nitrogen fraction of the bulb during the course of this developmental sequence. Table II has already indicated qualitatively the main sequence. In the figure the percentage of the total soluble nitrogen contributed by each amino-acid is represented in the form of histograms, adopting the convention that amides glutamine (No. 9), asparagine (No. 6), γ-methyleneglutamine (No. 29), and its free acid (No. 30) are plotted from the top of the histogram downwards; arginine (No. 13) is represented below these and the most important free amino-acids are represented from the base of the histogram upwards in the order aspartic (No. 2), glutamic (No. 3), γ-aminobutyric acid (No. 23), alanine (No. 8), serine (No. 4), and threonine (No. 7) followed by lysine (No. 12), proline (No. 15), valine (No. 16), and leucine(s) (No. 18). Amino-acids that could be detected as traces on the chromatograms but not quantitatively determined are listed only by number. In this way the course of the main changes in the relative composition of the alcohol-soluble nitrogen fraction may be seen, these are:

THE SOLUBLE NITROGEN COMPOUNDS OF TULIP: CHANGES DURING FLORAL INITIATION AND DEVELOPMENT

(WHERE NOT SHOWN NOS. 4,7,12,15,16,18,21,30, PRESENT IN TRACE AMOUNTS)



KEY TO FIGURE

2, aspartic acid; 3, glutamic; 4, serine; 6, asparagine; 7, threonine; 8, alanine; 9, glutamine; 12, lysine; 13, arginine; 15, proline; 16, valine; 18, leucine(s); 23, γ-aminobutyric; 29, γ-methyleneglutamine; 30, γ-methyleneglutamic acid.

(*a*) Until the flower was completely laid down and had time to develop toward maturity (Period 6) the composition of the alcohol soluble nitrogen in the bulb remained dominated by arginine. The amides glutamine and asparagine, which are often conspicuous in storage organs, e.g. potato tuber (Steward and Street, 1946), were conspicuous by their absence or very low content in the completely vegetative tulip bulb. (The same general picture was noted in Table II, namely, little or no asparagine or glutamine in the bulb prior to floral initiation and development.) Thus arginine plays the role of the most conspicuous soluble nitrogenous reserve substance in the bulb of the tulip.

(*b*) Though both glutamine and asparagine were virtually absent from the bulb prior to the maturation of the flower at 48° F., the γ-methyleneglutamic compounds (i.e. the amide and the free acid) occurred throughout this phase, although their contribution to the total soluble nitrogen was relatively small and surprisingly unaffected by the remarkable sequence of morphological changes between Period 0 to 6, which caused the relatively large changes in other nitrogenous constituents. Again, therefore, the γ-methyleneglutamic compounds in the tulip seem somewhat remote from the nitrogen metabolism that is most closely associated with growth and development and they appear as though superimposed upon a pattern of nitrogen metabolism which is mainly remarkable for the way in which arginine is emphasized in the metabolism of the bulb and the more conventional amides do not appear conspicuous until after flowers have formed and they and the aerial shoot has developed. Other examples in which arginine takes over a role otherwise attributed to amides are well known, as in the cases cited by Chibnall (1939).

(*c*) When the floral shoot with its photosynthetic leaves is ready to emerge from the bulb the emphasis on arginine in the soluble nitrogen content of the bulb was replaced by a soluble nitrogen pool in which glutamine, asparagine, and a variety of free amino-acids occur, but again the γ-methyleneglutamic acid compounds remained relatively inconspicuous.

SUMMARY

1. By qualitative and quantitative procedures based on paper partition chromatography, the nitrogen compounds of the tulip bulb have been investigated at various stages of development.

2. Prior to floral initiation the main constituent of the alcohol-soluble nitrogen fraction is free arginine and 47 per cent. of nitrogen of the alcohol-insoluble fraction may be accounted for, after hydrolysis, as arginine.

3. Minima in the content of total soluble nitrogen occur at peak periods in the development but, as floral initiation and development proceeds, the soluble nitrogen fraction is replenished from the arginine-rich insoluble fraction.

4. The nitrogenous economy of the bulb revolves around arginine, that of the leaf around the amides (notably glutamine) as the principal soluble compounds. The remobilization of the nitrogen compounds and the shift from a preponderance of arginine, as in the bulb, to a preponderance of amide

nitrogen in the soluble fraction, as in the leaf, does not occur till after floral initiation is complete and the flower develops.

5. The γ-methyleneglutamic compounds, i.e. the amide and free acid, seem to be remote from and superimposed upon the changes most concerned with the course of development in the bulb. These compounds appear to be surprisingly unaffected by the morphological events involved in floral initiation and development. This is not so surprising when it is realized that the high content of γ-methyleneglutamine in the tulip, *T. gesneriana*, is not a characteristic of all species of *Tulipa* but is peculiar to only a few. The accumulation of these compounds, therefore, does not appear to play an essential role in the development of the tulip but it again appears to be the result of some gene determined changes in the metabolism which have occurred during breeding, selection, or evolution, and which is now superimposed upon the more fundamental pattern of nitrogen metabolism. In the latter the substances arginine on the one hand and the amides (glutamine and asparagine) on the other play more intimate, characteristic, but contrasted roles.

ACKNOWLEDGEMENTS

This work was done in two parts and the first results of it were described in the Ph.D. Theses of two of us (R. M. Z. and H. M. C.). In that part of the work carried out in the University of Rochester (by R. M. Z. and F. C. S.), acknowledgement should be made to the Rochester City Parks for supplies of tulip bulbs. In that part of the work which represents a collaboration between the Departments of Botany and Floriculture at Cornell University (by H. M. C. and F. C. S.) acknowledgement should be made of the help furnished by the Department of Floriculture through the interest of the late Dr. Kenneth Post in the problem.

The chromatographic work at Cornell was also facilitated by help furnished with the aid of the grant from the Grasselli Chemicals Department of the E. I. du Pont de Nemours & Company, Inc. to Cornell University for work being directed by one of us (F. C. S.).

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The Spatial Development of *Spartina* Colonies Growing without Competition

BY

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With Plates X and XI and seven Figures in the text

Abstract

The spatial development of circular auxoclones of *Spartina townsendii* colonizing bare mud or muddy sand is described. It is shown that enlargement of the auxoclones is accompanied by a change in shoot density distribution and development of a concentric pattern. Additional 'rings' of high shoot density are added as the auxoclone increases in diameter and those in the centre are maintained. The number of rings is proportional to the diameter, and auxoclones reached the same complexity at the same diameter on different substrates. This type of development is discussed in relation to *Spartina* morphology.

I. Introduction

THE results described in this paper were obtained between July 1954 and October 1955. Most of the data were collected at Blakeney Point, Norfolk, but they have been extended by observations in the Tamar estuary, Devon, and at Dawlish Warren in the Exe estuary, Devon.

The 'backbone' of Blakeney Point is a shingle spit, separating from the mainland at Weybourne and running out in a west-north-westerly direction at a slight angle to the mainland coast. Lateral shingle banks run south from the main spit, and are separated from the mainland by Blakeney Channel (Text-fig. 1). Between these banks are open mud and muddy sand flats, which are being colonized by small circular colonies of *Spartina townsendii*. A general view of one of these areas is shown in Plate X*a*.

These circular colonies are growing without competition (except where overcrowding leads to fusion of adjacent colonies), and this paper is concerned with the spatial development of such colonies as they enlarge. Preliminary observations suggested that enlargement is accompanied by a change in shoot density distribution, and the development of concentric pattern. Colonies with a diameter of about 115 cm. show a central area of low shoot density, surrounded by a ring of high shoot density, while slightly larger colonies show an apparent regeneration in the central area, which now has a high shoot density and is separated from an outer ring of high shoot density by a ring of low shoot density (Plates X*a* and *b*).

It was decided to investigate these density relationships quantitatively.

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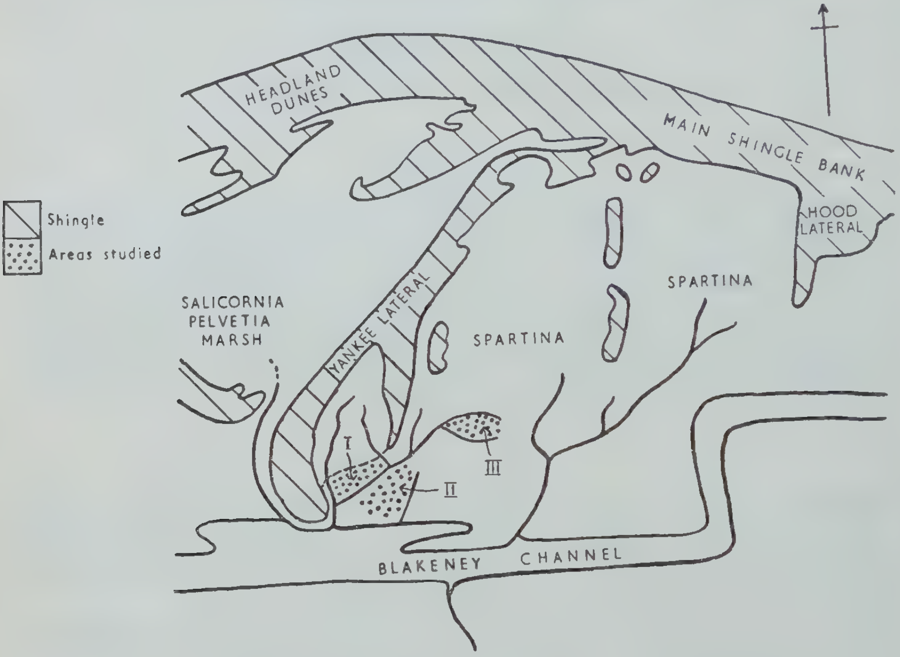
II. METHOD

(1) *Analysis of Colonies*

A ladder of contiguous quadrats was constructed, and placed across the diameter of a colony. Each quadrat was 25 cm. across and 15 cm. long, the total length of the ladder being 150 cm. In each quadrat the number of shoots was counted, and the diameter of the colony was measured. The colonies in three areas close to the south-eastern tip of Yankee Bank lateral were chosen for analysis. All the colonies in each area were analysed, 32 in Area I, 29 in Area II, and 56 in Area III. These areas are marked in Text-fig. 1. Soil profiles were dug inside and outside colonies in each area. Colonies in Areas I and II had rhizomes running in mud, those in Area III had rhizomes running in muddy sand.

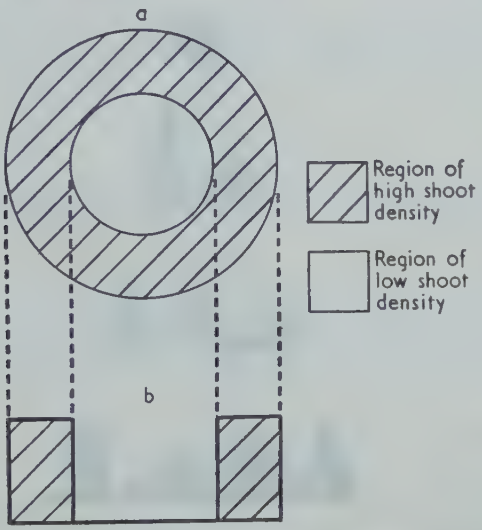


TEXT-FIG. 1. Map of Blakeney Point. Location of the areas studied.

The results of the quadrat counts in each colony were expressed in histogram form. Thus a colony showing an outer ring of high shoot density surrounding a central area of low shoot density (Text-fig. 2*a*, and Plate X*b*), would have a histogram such as is shown in Text-fig. 2*b*.

(2) *Classification of Histograms*

When examined, the histograms showed a number of well-defined zones of high and low shoot density. Difficulty was found in devising a perfectly objective method of classification, however, since the overall vigour of growth varied from colony to colony, e.g. two colonies might show the same shoot density distribution, but whereas in a vigorous colony the high density zone might be represented by quadrats containing 50 shoots per quadrat and the low density zone by quadrats containing 10 shoots per quadrat, in a colony of poorer growth the quadrats of the high density zone might contain 25 shoots per quadrat and those of the low density zone 3 shoots per quadrat. This factor precluded any rigid mathematical classification. A number of methods were tried, but it was decided finally that the simplest and most objective method was to count the number of high shoot density zones shown on the histograms and classify the colonies accordingly (Table I).



TEXT-FIG. 2. *a* Aerial view of an ideal Class 2 colony. *b* Diagram of Class 2 colony histogram, ignoring quadrat grouping.

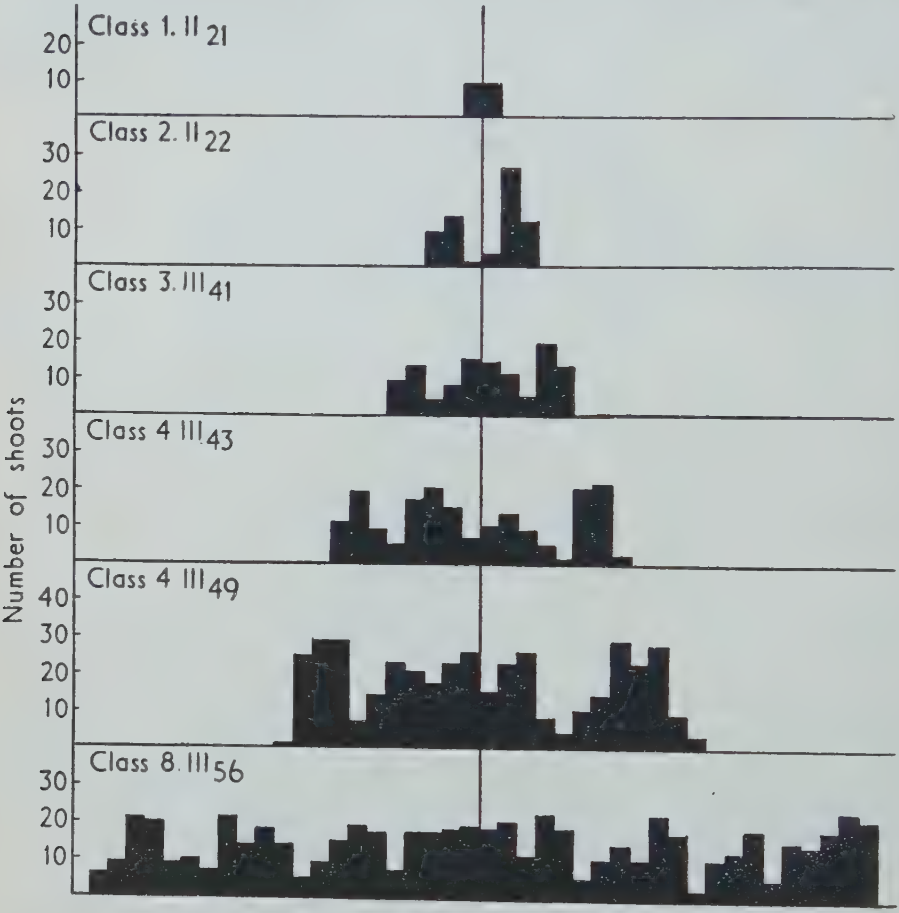
TABLE I

Class	Number of zones of high shoot density shown in histogram
1	1
2	2 (Photograph 2)
3	3 (Photograph 3)
4	4
5	5
6	6
7	7
8	8

Thus the type of colony shown in Text-fig. 2 would be a Class 2 colony. A Class 4 colony would have 2 rings of high shoot density separated by a ring of low shoot density, the inner ring of high shoot density enclosing a central zone of low shoot density.

Typical histograms are shown in Text-fig. 3.

The class of each colony was plotted against its diameter. The results are shown in Text-fig. 4*a* (colonies with rhizomes running in muddy sand), and Text-fig. 4*b* (colonies with rhizomes running in mud). Few colonies of the higher classes were analysed, (*a*) because few colonies in the areas studied had reached these complexities, and (*b*) most of those which had reached these complexities had fused with adjacent colonies. This means that average diameter figures for colonies of higher classes are subject to greater error than those of lower class colonies.



TEXT-FIG. 3. Typical histograms.

III. *Results*

Text-figs. 4*a* and *b* show that on the average the Class of a colony is proportional to its diameter, i.e. a particular shoot density distribution is characteristic of a particular diameter. Thus as the colony enlarges its shoot density

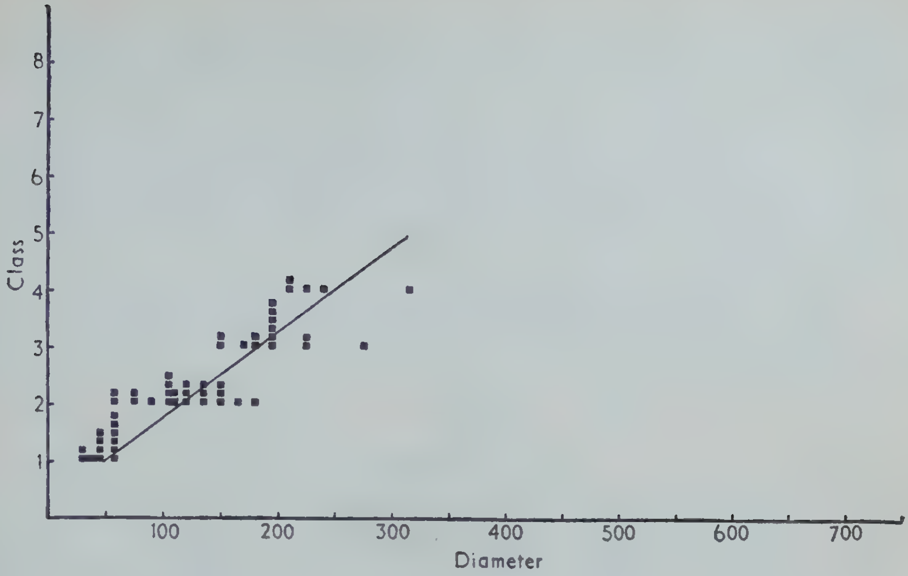


FIG. 4*a*

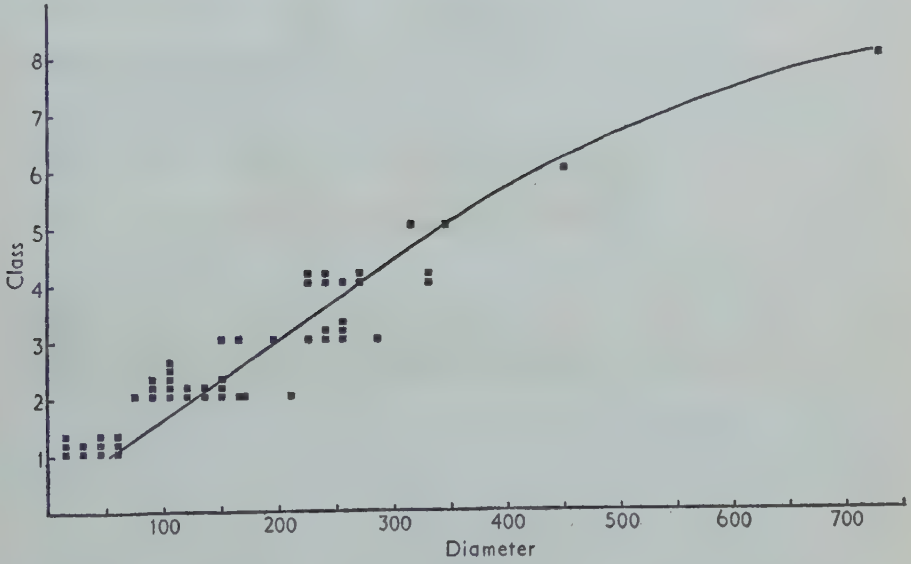


FIG. 4*b*

TEXT-FIG. 4. Class × Diameter. (*a*) Colonies from Areas I and II (rhizomes running in mud). (*b*) Colonies from Area III (rhizomes running in muddy sand).

distribution alters, and an increasing number of concentric rings is added to it. Further, in colonies where comparable figures are available for colonies with rhizomes running in mud, and in muddy sand, colonies of the same diameter have reached the same complexity, regardless of substrate. This, however, does not necessarily mean that colonies of the same complexity and diameter are of the same age on mud or muddy sand, since they may be growing at different rates on different substrates. Preliminary evidence suggests that colonies on muddy sand were increasing in diameter faster than those on pure mud.

Analysis of the data confirms that there is a change in shoot density distribution, and the addition of concentric rings of high shoot density as the colonies enlarge.

IV. *Morphology*

Most of the data in this section have been supplied by Mr. P. Goodman, to whom the author wishes to express gratitude.

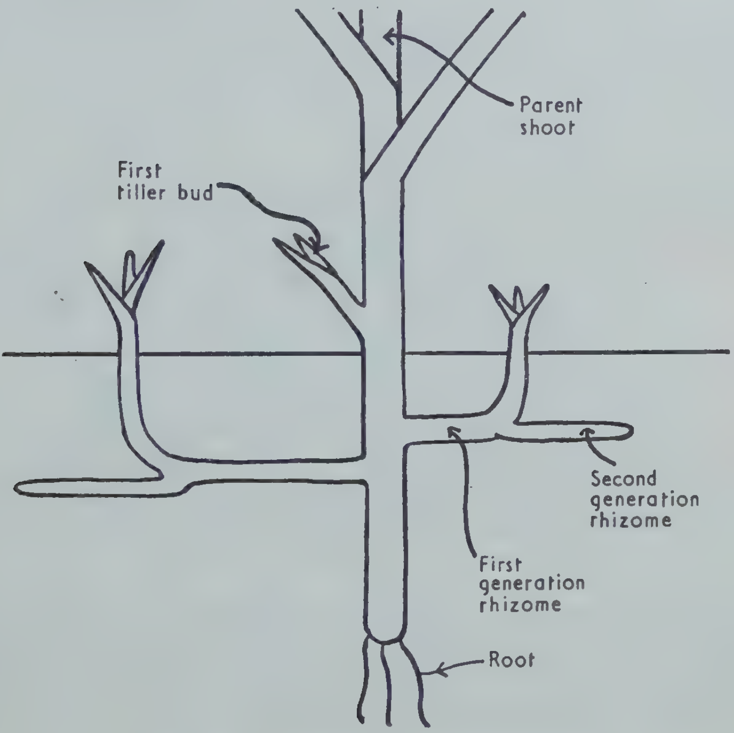
The *Spartina* seedling has a basal rosette of leaves, each with a bud in its axil, borne on nodes separated by short internodes. These buds may develop into tillers or rhizomes. Evans (1927) defines a tiller as an intravaginal shoot, green and leafy, and a rhizome as an extravaginal shoot which is white and scaly.

The lowest buds on the parent stock give rise to rhizomes and those further up to tillers or short rhizomes. In *Eriophorum*, Phillips (1953) has shown that the formation of rhizomes from downwardly directed buds low on the stock, and of tillers or short rhizomes from buds higher up on the stock, is a gravitational effect.

The stock of the *Spartina* seedling may put out a rhizome from the bud in the lowest axil; or tiller first and then put out a rhizome from a bud in a low stock or tiller axil. More rhizomes may then follow from other low axils. These may be called the first-generation rhizomes and since the phyllotaxy in *Spartina* is $\frac{1}{2}$, their buds are opposite, and on the upper and lower faces of the rhizome. After the production of a few internodes, the first-generation rhizomes turn upwards close to the parent stock and outward growth is continued by second-generation rhizomes arising from buds on the lower and outwardly directed side of the first-generation rhizomes. If it has not already done so, the parent stock now tillers (Text-fig. 5).

The second-generation rhizomes grow out strongly while tillers form from the upper buds on the parent stock, and also from buds on the uppermost side of the first-generation rhizomes. Thus a shoot-clump is formed, consisting of the parent stock plus its associated tillers and the first-generation rhizomes plus their associated tillers. From this shoot-clump radiate out the second-generation rhizomes. In the light of the evidence from *Eriophorum*, it is very tentatively suggested that the production of tillers from buds on the uppermost side, and rhizomes from buds on the lowermost side of the first-generation rhizomes may be a gravitational effect. The plane of the buds on the second-generation rhizomes will be horizontal, and therefore there will be no difference in the gravitational force exerted on the two ranks of buds. If the buds of the second-generation rhizomes grow out they form rhizomes.

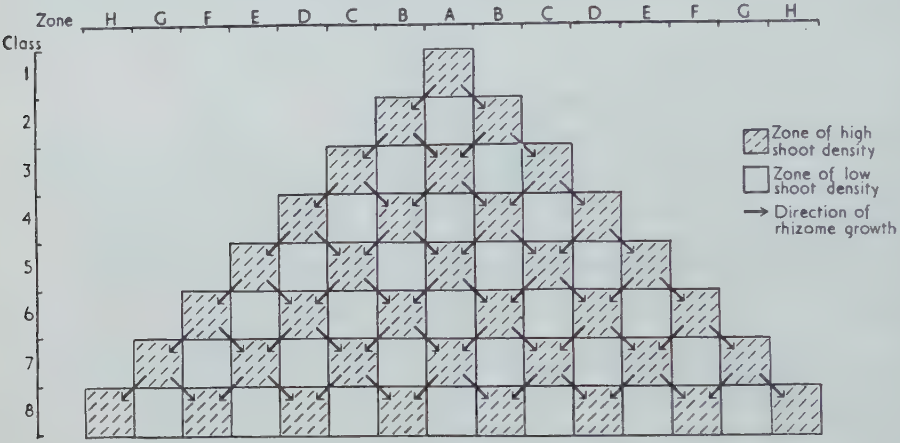
According to Goodman, the parent shoot must produce 10 leaves prior to flowering, but once it has flowered, 'facilitation' of flowering of the associated tillers occurs, i.e. they can flower with only 6–8 leaves. If a shoot arises too late to raise the necessary number of leaves prior to flowering in the same season, it remains dormant until the following season. After flowering and seed ripening, the stem dries up and breaks off at the base leaving a stump.



TEXT-FIG. 5. Diagram of young shoot-clump.

A young colony consists of a central shoot-clump with second-generation and third-generation rhizomes radiating out from it. The third-generation rhizomes arise from buds on the second-generation rhizomes. The second- and third-generation rhizomes turn up and form new shoot-clumps round the periphery of the colony.

Evans (1927) in his work on *Phleum*, defines a plant as 'all growing shoots developed from a single seed or vegetative part, even if the intervening parts have disintegrated'. Since in *Spartina* colonies may exceed 12 ft. in diameter, it seems misleading to refer to them as plants. Further, the term 'colony' is unsatisfactory, since a colony may consist of individuals of more than one genetic origin. After some consideration it was decided to introduce a new term, and the words polyclone and auxoclone suggested themselves. *Auxoclone* was finally chosen since, whereas a polyclone would indicate a group of individuals (shoot-clumps in the case of *Spartina*), of the same genetic origin, an auxoclone refers to an enlarging or increasing group of individuals of the same genetic origin. It is thought that the term might be useful as a general one to describe any type of discreet group spreading vegetatively by addition of individuals, since it does not imply any particular size or form of group, or method of vegetative spread, but is limited to an enlarging clone.


Zone
H
G
F
E
D
C
B
A
B
C
D
E
F
G
H
Class
1
2
3
4
5
6
7
8
Zone of high shoot density
Zone of low shoot density
Direction of rhizome growth

TEXT-FIG. 6. Diagram of transections across successive classes of auxoclones.

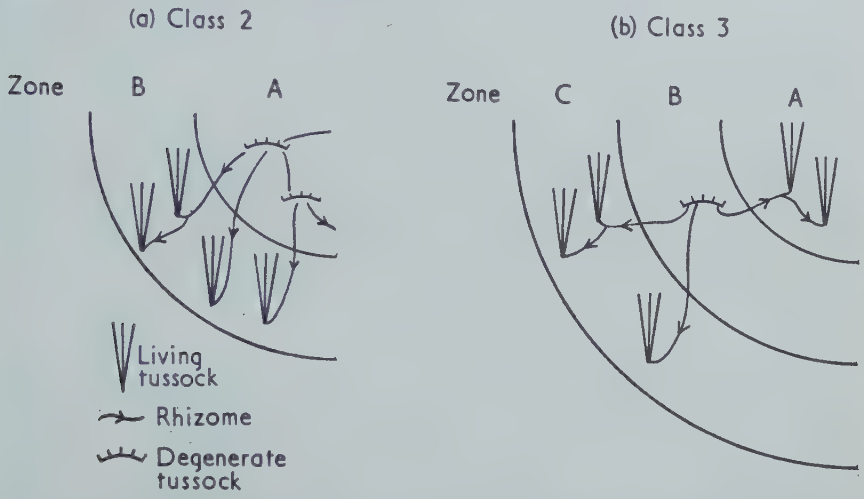
In *Spartina* the rhizomes and shoot-clumps persist for a long time, possibly due to the anaerobic nature of the soil, so that by scraping away the accumulated sediment it is possible to trace the successive directions of growth of several generations of rhizomes and shoot-clumps.

V. *Discussion*

Developing auxoclones acquire a ring pattern, and as they enlarge further concentric rings are added on. If the arrows are for the moment disregarded, Text-fig. 6 shows diagrammatically transections across successive classes. For reference the zones of development have been lettered. The notation used will be the Class number, followed by the Zone letter. Thus the B zone of a Class 2 auxoclone will be referred to as 2 zone B, or the A zone of a Class 3 auxoclone as 3 zone A.

In order to investigate the causes of orderly development of concentric pattern, shoot-clumps from various zones of different classes were dug up, and the rhizome systems of Classes 1, 2, and 3 examined in more detail.

Class 1 auxoclones have an even distribution of shoots throughout. In Class 2 auxoclones, this is replaced by 2 zone A which has a low shoot density compared with 2 zone B, for in many cases the former may contain only two or three shoots. If the rhizome system of a Class 2 auxoclone is exposed by clearing away the mud or muddy sand 2 zone A is found to be occupied by degenerate shoot-clumps with only the stumps of the shoots remaining. From these shoot-clumps rhizomes radiate out to 2 zone B where they give rise to young living shoot-clumps (Text-fig. 7*a*).



TEXT-FIG. 7. Diagrams of exposed rhizome systems. (*a*) Exposed rhizome system of a Class 2 auxoclone. (*b*) Exposed rhizome system of a Class 3 auxoclone.

Thus the transition from Class 1 to Class 2 type involves the shoot-clumps of 1 zone A sending rhizomes outwards into any available space, i.e. into the potential 2 zone B. These rhizomes turn up and tiller, giving the high shoot density characteristic of 2 zone B, while the shoots on the shoot-clumps of 1 zone A flower and produce seed, subsequently degenerating and breaking off at the base leaving stumps. The relic shoot-clumps of 1 zone A become buried under accumulating silt, giving the low shoot-density zone found in the centre of Class 2 auxoclones, 2 zone A.

Class 3 auxoclones have a central zone of high shoot density, 3 zone A, separated from an outer ring of high shoot density, 3 zone C, by a ring of low shoot density, 3 zone B. The problem to be considered then is the origin of the apparent regeneration in 3 zone A. This might arise from one or more of three sources, i.e. from seed, by a true regeneration from relics of 1 zone A, or from the invasion of zone A by rhizomes growing inwards from 2 zone B.

Since no seedlings have ever been found in 3 zone A, even in the early stages of its formation, origin of the high shoot density characteristic of this zone from seed seems unlikely.

In 3 zone A decayed shoot-clumps are sometimes found bearing one or two new shoots. There do not seem to be a sufficient number of these to account for the large number of shoots in 3 zone A, and (referring back to the morphology), it seems more likely that these are shoots which have arisen too late in the season to raise the necessary number of leaves prior to flowering, so that while the rest of the shoot-clump has flowered and subsequently degenerated, these shoots have remained dormant until the following season.

Inward growth of rhizomes from shoot-clumps of 2 zone B appears to be the main source of origin of the high shoot density characteristic of 3 zone A. Exposure of the rhizome system of a late Class 2 auxoclone at Blakeney showed rhizomes starting to grow back from 2 zone B towards 2 zone A (potential 3 zone A). At Dawlish, digging up 3 zone A of an early Class 3 auxoclone showed that it was filled with rhizomes starting to turn up and tiller, leading in from zone B. These inwardly growing rhizomes may be seen in Plate XI*b*. Further, at Blakeney, by digging in 3 zone B, it was possible to find relic shoot-clumps with rhizomes leading outwards and in connexion with shoot-clumps of 3 zone C, and also, from the same shoot-clumps, rhizomes leading inwards, and in connexion with shoot-clumps of 3 zone A (Text-fig. 7*b*).

Transition from Class 2 to Class 3 involves the outgrowth of rhizomes from shoot-clumps in 2 zone B into any available space, i.e. outwards to give rise to 3 zone C, and inwards into zone A (the low shoot-density zone of Class 2 auxoclones), to give rise to the high shoot-density zone A of Class 3 auxoclones. Meanwhile, the shoots on 2 zone B shoot-clumps flower and subsequently degenerate, leaving the relic shoot-clumps of 3 zone B, which become buried by accumulating silt.

Looking at Fig. 6 where the arrows indicate the direction of rhizome growth it is possible to see how, by the continuous process of rhizoming, tillering and degeneration of shoot-clumps following flowering, coupled with the production of more rhizomes invading further space, successive concentric circles of high shoot density may be added onto the periphery of an auxoclone, while those nearer the centre are maintained.

In the lower classes of auxoclone (Classes 1–4) concentric zonation is usually clearly visible to the eye, but in the more complex classes it may only be revealed by analysis since the zones tend to become less distinct.

Watt (1947*a*) as a result of his studies on *Pteridium* and other plant communities, postulates that the community exists in a dynamic state, both spatially and temporally. He demonstrates the existence of four phases—pioneering, building, mature, and degenerate. The *Spartina* auxoclones described above may be easily fitted into this scheme, the whole cycle being continuously repeated in any one zone. Thus the pioneering phase consists of the invasion of the zone by young rhizomes. During the building phase the rhizomes turn upwards and tiller forming shoot-clumps. In the mature phase the shoot-clumps shoots flower, and following this degenerate, leaving a space clear for reinvasion by another generation of young rhizomes.

Spartina only shows this regular concentric development where it is growing without competition and has space to spread undisturbed in any direction.

Thus it does not show it at Blakeney where it is colonizing the restricted space available along channel edges in the upper marshes. It is interesting to note that a number of other plants show ring formation where they are in a position to spread in any direction.

1. *Phleum pratense* L.

Evans (1927) describes the vegetative spread of shoot-clumps transplanted in rows, 3 ft. 3 in. apart. The plants enlarged to form a circle, and during the first two seasons the whole of the area was occupied by a dense growth of living shoots. Evans goes on to say that by the fifth season, in most plants the centres were occupied only by dead roots and stubble, while the periphery consisted of a dense ring of living shoots suggesting 'fairy rings'. Evans says that *Phleum* plants do not increase in size by means of true rhizomes, but slow increase in size results from the outward growth of procumbent proaxes. Rhizomes only develop under abnormal conditions.

2. *Luzula sylvatica* Hirds.

Auxoclones of *Luzula* growing in a beechwood on a steep escarpment facing west at Noss Mayo, South Devon, were examined. Here there was little competing ground flora, and those auxoclones in which the slope did not interfere with spatial spread in any direction showed concentric development of the Class 2 *Spartina* type. In auxoclones of about 60 cm. diameter the central bare zone was surrounded by a ring of high shoot density. Exposure of the rhizome system showed a central degenerate shoot-clump from which three rhizomes radiated out, each branching once and terminating in a peripheral shoot-clump.

3. *Triglochin maritima* L.

Two vigorous auxoclones were found growing on bare mud at the upper limit of the intertidal zone on the south side of Millbrook Creek in the Tamar estuary. These also showed Class 2 type development.

4. *Agrostis canina* and *A. tenuis*.

Watt (1947*a*) describes the development of rings by the vegetative spread of a single plant. Dead shoot-clump remains are found beneath the mat of *Cladonia* which develops in the central area of low shoot density.

5. *Pteridium*.

Watt (1947*b*) describes the colonization of grass heath by bracken. Normally invasion takes place along a linear front but he describes the occasional occurrence of circular auxoclones similar to those mentioned above. Further, regeneration may occur in the central low shoot-density area, but unlike *Spartina*, this may be from relics as well as from inwardly growing pioneer rhizomes.

In addition to the plants noted above which have been investigated, there is evidence that the following plants may also show concentric zonation.

1. *Bromus inermis.*
2. *Eriophorum vaginatum.*
3. *Molinia coerulea.*
4. *Schoenus nigricans.*
5. *Nardus stricta.*

Thus the early stages of development of the concentric pattern described for *Spartina* seem to be characteristic of plants with a branching rhizomatous system (or a semi-rhizomatous system as in *Phleum*), and are best shown where there is little or no competition and the plant is free to develop in any direction. The reinvasion of areas of low shoot density formed by degeneration of old shoot-clumps does not appear to be so characteristic, occurring only in *Spartina* and to a limited extent in *Pteridium*. It is not yet clear whether this is due to absence of data for the later stages, or whether in fact there is no further development in the centre. In *Agrostis* (Watt, 1947*a*) the central zone of low shoot density may be colonized by a different plant. *Spartina* appears to be unique in the development and duration of complex concentric zonation.

ACKNOWLEDGEMENTS

Most of the work described in this paper was carried out while the author was a student in the Botany Department, University College, London. The author wishes to express her thanks to Professor Pearsall and Mr. P. J. Syrett for advice and encouragement, and also to Mr. P. Goodman of Southampton University for helpful discussion and permission to publish the data on *Spartina* morphology and Plate XI*b*.

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EXPLANATION OF PLATES X AND XI

PLATE X

Fig. *a*. General view of Area II. In the foreground are four fusing auxoclones, and behind, scattered circular auxoclones.

Fig. *b*. Class 2 auxoclone showing ring of high shoot density enclosing a central bare zone.

PLATE XI

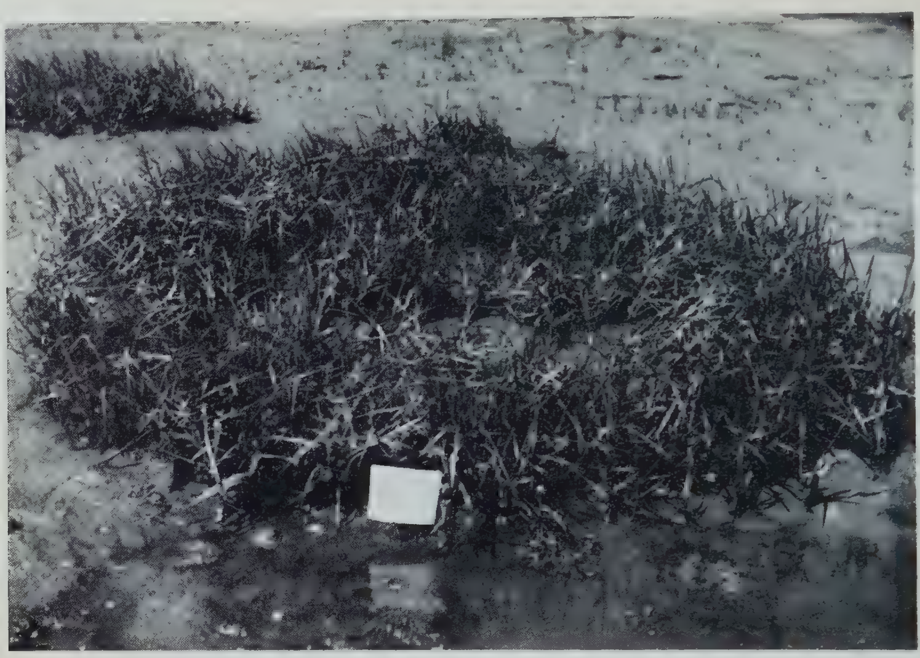
Fig. *a*. Class 3 auxoclone showing apparent regeneration in central zone.

Fig. *b*. Late Class 2 auxoclone. The photograph shows the exposed decayed tussocks of the central bare zone, being invaded by young rhizomes from the outer zone of high shoot density.

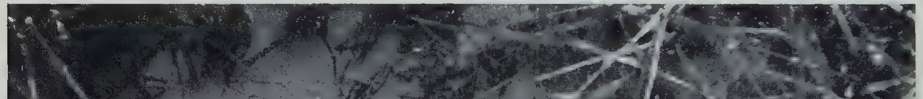
a

b

P.-A. CALDWELL



a



b

P.-A. CALDWELL

Fusarium Wilt of Bananas in Jamaica

II. Some Aspects of Host-parasite Relationships

BY

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With one Figure in the Text

ABSTRACT

The incidence of *Fusarium* wilt in a given variety of banana is determined by numerous interacting factors such as genetically determined resistance, physical and chemical soil conditions, and abundance of *Fusarium oxysporum f. cubense*. To attain marked resistance to wilt, 'Gros Michel' apparently requires a combination of conditions; thus the soil must be alkaline, of medium to heavy texture, and rich in available potash. Nitrogen fertilizing tends to increase susceptibility to wilt.

Some striking effects of weather on wilt incidence are described and various aspects of crop management are discussed: soil deterioration under bananas undoubtedly contributed to development of wilt in the past.

In the earliest stages of rootlet infection, the pathogen grows more slowly in the resistant 'Cavendish' than in the susceptible 'Gros Michel', probably owing to more effective tissue reactions in the former. In the field, infections progressing along the root are often checked at the root base: with 'Gros Michel', however, a small proportion enter the rhizome and as a result wilt generally develops. Ability to check such infections, leading to recovery, is only exhibited on very fertile soils.

The possible mode of action of various factors on wilt incidence is discussed, and the importance of vigorous root growth in promoting host resistance is emphasized.

FACTORS AFFECTING INCIDENCE OF WILT

i. *Genetic constitution of the host*

IT has long been known that some banana varieties are relatively resistant to wilt: foremost amongst these in the West Indies are the Cavendish group (Simmonds, 1954), which includes 'Lacatan', 'Robusta', and the less commonly grown 'Cavendish'. In Jamaica 'Lacatan' grows well at a variety of sites where earlier 'Gros Michel' was severely affected by wilt and its resistance is undoubtedly high. Resistance to wilt is determined genetically, and for some years one of the main objects of the West Indian Banana Research Scheme has been to breed wilt-resistant varieties having the desirable characters of 'Gros Michel'. Many recently bred seedling varieties —tetraploids—in fact show marked resistance to the disease. In all probability, no variety is entirely immune to *Fusarium oxysporum f. cubense* (E. F. Smith) Snyder and Hansen: at various times wilt has been recorded for most resistant varieties

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and there is evidence that these suffer a certain amount of root infection, though wilting is rare.

In the current investigation it was found that resistance to *F. oxysporum f. cubense* could be demonstrated by means of a pot test. Small suckers were grown in earthenware pots containing unsterilized light loam and were inoculated with a sawdust/maize-meal culture of the fungus, as described earlier (Rishbeth, 1955). In a preliminary experiment a series of 10 'Gros Michel' plants were found to have a total of 38 rhizome infections after 3 months' growth, whereas a comparable series of 'Lacatan' had only 1. In a further experiment 2 plants each of a number of varieties, chiefly seedlings, were similarly tested. Four varieties, including 3 known to be susceptible in the field, had an average of 8·4 infections per rhizome, whereas 12 varieties known to be resistant had an average of 0·2 infections. When this experiment was repeated on a somewhat larger scale very similar results were obtained. There was thus excellent agreement between susceptibility to wilt based on Jamaican field experience and susceptibility to rhizome infection as judged by the pot test. Such a test may prove useful for routine testing of seedling varieties since results are obtained more quickly than with a field test. However, where only a limited number of suckers are available, it would be impossible to establish differing degrees of resistance. (It might prove practicable to make such a test independent of laboratory culture by using as inoculum thoroughly disintegrated infected pseudostem; ordinarily chopped pseudostem is, however, inferior to a sawdust/maize-meal culture as inoculum.)

ii. *Physical and chemical soil conditions*

Banana wilt occurs under such a wide range of soil conditions that it is not easy to ascribe special significance to any one factor. It is well known in the Caribbean area that the disease is prevalent in situations subject to temporary waterlogging, and a similar association has been reported in Malaya (Ward, 1930) and Ceylon (Fernando, 1940). In Surinam, Drost (1912) drew attention to the increased incidence of banana wilt associated with a deepening of drainage ditches: this he attributed to the resulting greater fluctuation in soil moisture content. In the Caribbean area, texture is generally recognized as being important, wilt tending to be more severe on light-textured than on heavy soils, provided that the latter are well drained. Croucher and Mitchell (1940) found that in Jamaica, under similar conditions of infection, the disease spread more rapidly in acid soils (below pH 5·5) than in slightly acid or alkaline soils. Wardlaw (1941) sums up the position for Central America by stating that hydrogen-ion concentration is the master factor determining severity of the disease, followed by texture. Evidence of the possible effect of soil nutrient status on wilt incidence is meagre and somewhat conflicting. Smith (1932) goes so far as to say that the better the cultivation and the richer the land, the more rapidly wilt spreads.

Despite the widespread destruction caused by banana wilt in Jamaica, scattered plantations of 'Gros Michel' were still to be found in 1950–2. These varied considerably in health, some having remained virtually free from disease for 20 years or more, despite ample opportunity for infection, whereas others had suffered a rapid and severe attack of wilt. It was thought that a survey of representative plantations might provide evidence of the conditions necessary for wilt resistance and accordingly visits were made to record wilt incidence and to investigate soil conditions. Information about the stature and fruit yield of the bananas, the method of cultivation, and the history of the site was also collected. Bulk samples were taken from the first 15 cm. of the soil profile, from a position between the plants to avoid an area to which in some instances fertilizers had been applied. Texture was assessed in the field and further analysis was carried out by the Agricultural Chemist, Department of Agriculture, Jamaica, the methods being as follows (Piper, 1942). Soil reaction was determined on 1:2·5 soil:water suspensions by means of a quinhydrone electrode. Organic matter was estimated by Walkley and Black's rapid titration method, values for percentage organic carbon being multiplied by the conventional factor 1·72. Available phosphate was estimated by a modification of the Lorenz method, colour being determined with a photoelectric colorimeter, and available potash by the volumetric cobalti-nitrite method after leaching the soil sample with 0·5N acetic acid. The findings are perhaps best conveyed by comparing plantations virtually free from wilt and over 20 years old with those severely affected within 2–3 years of planting. With the latter, attention was confined to sites where wilt had appeared since 1948: in the case of earlier outbreaks it was difficult to ascertain just how rapidly wilt had developed. Table I records the number of soil samples falling into various categories and also gives the former status of the site. For convenience, upland sites having mainly sedentary soils are separated from lowland ones having sedimentary soils.

In commenting on these results, allowance has to be made for the relatively few plantations of each type visited and for the limited value of deductions based largely on soil analyses. The importance of soil texture is apparent: thus plantations on light-textured soils are often severely affected. Certain clays, particularly those exposed by erosion, impede drainage and allow only poor root growth: under such conditions 'Gros Michel' readily succumbs to wilt. Other clays, better aerated and draining more readily, are apparently more suitable for healthy growth of this variety, probably because moisture content fluctuates less than in lighter soils. It is probably significant that the great majority of healthy plantations in hill country are found on medium to heavy loam overlying clay. On the generally free-draining sedimentary soils, the lightest texture which will support healthy 'Gros Michel' for over 20 years is a medium loam. One point not brought out by Table I is the importance of aeration: healthy plantations invariably had at least 30 cm. of well-aerated surface soil.

The tendency for banana wilt to be less severe on alkaline soils than on acid ones is clearly illustrated by plantations in hill country: over 70 per cent. of soil samples from healthy plantations had a pH value in the range 7·6–8·5, whereas no soil sample from severely affected plantations fell in this range. The fact that the great majority of samples from plantations on sedimentary soils were alkaline, irrespective of wilt incidence, is due to the preponderance of limestone in Jamaican hill country. Plantations on alluvium remained almost free from wilt long after the disease was established in the uplands, and this was earlier assumed to provide another instance of the relative resistance of bananas on alkaline soils. However, it seems probable from present knowledge of alluvial soils that texture was often too light and nutrient status too low for bananas to have been appreciably resistant to wilt. This is borne out by the rapid and widespread outbreaks of wilt following the first serious floods. Here, initial freedom from the disease was probably due to absence of the pathogen.

TABLE I

The Relation between Soil Conditions and Wilt Incidence in 'Gros Michel': the Number of Diseased and Healthy Plantations falling into various Categories

SOILS
U Upland, mainly sedentary
L Lowland, sedimentary

PLANTATIONS
D Severely diseased within 2–3 years of planting
H Virtually free from wilt and over 20 years old

	TEXTURE				REACTION (pH)						
	Loam			Clay							
	Light	Medium	Heavy		5·1 to 5·5	5·6 to 6·0	6·1 to 6·5	6·6 to 7·0	7·1 to 7·5	7·6 to 8·0	> 8·1
UD	1	1	—	6	6	8	7	1	1	—	—
UH	—	7	7	2	—	—	—	2	5	9	18
LD	10	4	1	—	—	—	1	2	4	16	24
LH	—	7	6	—	—	—	1	5	2	12	16

	ORGANIC MATTER (%)						AVAILABLE PHOSPHATE (p.p.m. P_2O_5)					
	1·1 to 2·0	2·1 to 3·0	3·1 to 4·0	4·1 to 5·0	5·1 to 6·0	> to 6·1	1 to 20	21 to 50	51 to 100	101 to 400	401 to 1,000	> 1,000
UD	—	3	6	6	—	1	6	13	3	1	—	—
UH	2	2	6	8	—	5	—	11	8	14	3	3
LD	7	14	9	2	1	—	3	10	6	22	5	—
LH	1	5	4	7	4	6	—	—	3	11	17	6

	AVAILABLE POTASH (p.p.m. K_2O)								FORMER STATUS OF SITE		
	21 to 50	51 to 100	101 to 150	151 to 250	251 to 500	501 to 800	801 to 1,200	> 1,200	Sugar factory	House	No building
UD	1	2	2	7	8	—	—	—	—	—	8
UH	—	—	3	16	6	10	3	2	4	3	9
LD	2	10	10	16	7	1	—	—	—	—	15
LH	—	—	—	3	8	14	5	6	13	—	—

With sedentary soils there is no suggestion that wilt incidence is related to percentage organic matter in the soil. With sedimentary soils, however, severe wilt is often associated with a medium to low content of organic matter, a common feature of such soils when they are not bearing sugar cane. Several planters returned to cultivation of 'Gros Michel' on sites which had borne cane for periods up to 10 years, but the plantations were soon affected by wilt. This suggests that a temporary increase in the amount of organic matter is alone insufficient to affect wilt incidence.

As far as is known, it has not previously been suggested that the phosphate and potash content of soils affects the incidence of banana wilt, although a high potash status is known to decrease the severity of other wilts, such as that of cotton. In Jamaica, failure to observe such a relation could be explained in the case of most earlier surveys by the absence of facilities for soil analysis; however, even with such facilities the relation might have been obscured by interaction with other factors. The situation at the time of the present survey was particularly favourable for demonstrating a possible correlation, since wilt had been prevalent so long that only the most resistant plantations still survived. Table I illustrates the tendency for healthy plantations to be found on soils richer in available phosphate and potash than those on which severely affected plantations are found. In hill country this effect was partly due to the association of healthy plantations with old house or factory sites, and in the lowlands it was wholly due to this association. Patches of healthy bananas on old house sites were seldom more than half an acre in extent but often provided striking contrasts with affected bananas nearby: in a typical instance the average soil reaction was pH 7·6, as compared with 5·8 on the adjacent hillside, and the soil was much richer in available phosphate and potash. Plantations on sites of former sugar factories were often larger, up to 5 acres in extent, and on alluvial soils they tended to be isolated, neighbouring land having gone out of banana cultivation through wilt. Although the factories had been abandoned for over 100 years and the sites had often borne bananas continuously for decades, the soil was usually very rich in phosphate and potash, this fertility doubtless being the legacy of vast accumulations of plant debris and wood ash. It was not possible to demonstrate any increase in wilt resistance after applying phosphate or potash to bananas on less fertile soils and therefore the evidence for a relation between these nutrients and wilt resistance is wholly circumstantial. Such evidence for the effect of phosphate is hardly greater than that indicated in Table I, whereas that for the effect of potash is supplemented by other observations on 'Gros Michel'.

There existed on alluvium several old plantations which, although starting to develop wilt in 1950, had remained healthy some 15 years longer than neighbouring plantations on a similar soil type. The former were found to be growing on soils relatively rich in potash: samples collected in the manner described often gave values of *c.* 400 p.p.m. K_2O, unusually high for alluvium not associated with former factory sites. Again, a narrow roadside plantation about 200 m. long showed an interesting gradation of wilt incidence: at one end the crop had been entirely destroyed, in the middle it was partially affected, and at the other end it was healthy and producing excellent fruit.

The soil was a heavy clay-loam of about pH 8 throughout, but there was a gradation of available potash ranging from 150 p.p.m. K_2O at one end to over 300 p.p.m. at the other. The gradation of available phosphate was less pronounced. These differences had apparently arisen from an unequal deposition of debris near a former sugar factory. Lastly, the effect of potash was evident in young plantations at the Banana Breeding Station, Bodles, where bananas had not previously been grown. The soil, an alkaline loam, was moderately uniform in texture but varied considerably in potash content. Table II gives the average value for available potash at various locations and the incidence of wilt 20 months after planting. Fields 1 and 2 were planted at the same time and the others subsequently at about yearly intervals, the fields being adjacent.

TABLE II

Wilt Incidence in Relation to Soil Potash Content in some Young 'Gros Michel' Plantations

Location		Available potash (p.p.m. K_2O)	Percentage of wilted plants
Fields 1 and 2	SE. corner	291	34
	NW. corner	1363	1
	Remainder	481	5
	Field 3	215	12
	Field 4	466	2

Fields 3 and 4 were each moderately uniform with respect to soil potash and the difference in wilt incidence is striking. Fields 1 and 2 were not uniform in this respect and the high proportion of diseased plants in the south-eastern corner, where soil potash was relatively low, considerably raises the average for the whole (9 per cent.). The reason for the difference in wilt incidence between Field 3 and the south-eastern corner of Fields 1 and 2 is obscure. That this relation between wilt incidence and soil potash content is not purely a local phenomenon is suggested by analysis of soils from 'Gros Michel' plantations in Central America (Dunlap, 1952). Thus at a site where bananas were still healthy after 10 years and at another where half the cultivations were in good production after about 20 years, soil potash was very high. In two plantations giving less than 5 years' production, soil potash was only half as great.

By contrast, nitrogen had a relatively deleterious effect on wilt incidence, as was shown by the results of fertilizer experiments with 'Gros Michel'. In an experiment set up at five sites in Jamaica by Garrett (unpublished data) in 1948, plants receiving a monthly application of 4 oz. ammonium sulphate generally succumbed to wilt earlier than plants not receiving this fertilizer. After 17 months, the total number of wilted plants at the five sites in plots receiving nitrogen was 993 and in those not receiving it 544. A similar effect was seen in the current investigation when 16 plants growing on an alkaline loam were inoculated 3 weeks after planting with cultures of *F. oxysporum* *f. cubense*. 4 oz. ammonium sulphate was given monthly to 8 plants and the remainder were not fertilized. Of 7 plants developing wilt within 9 months of planting, 6 had received nitrogen. A 'Gros Michel' which was similarly fertilized for 6 months and then inoculated with chopped infected pseudostem developed wilt after 9 weeks. On one large estate, fertilizing with ammonium sulphate was abandoned after a trial had been followed by a marked increase of wilt: fertilizing with sodium nitrate did not have this effect, apparently. The greater susceptibility to wilt of bananas receiving heavy applications of nitrogen has not previously been reported, so far as is known, but the effect is well established with other *Fusarium* wilts such as those of tomato (Walker and Foster, 1946) and cantalupe (Stoddard, 1942). With both these diseases the balance between nitrogen and potash is considered especially important, wilt being more pronounced in plants receiving high nitrogen or low potash. Environmental conditions known to favour vascular wilts caused by various biologic forms of *F. oxysporum* have been conveniently tabulated by Garrett (1956).

Soil conditions similarly affect wilt incidence in banana varieties normally resistant to the disease. Of four minor outbreaks investigated in 'Lacatan', all were in plantations on heavy clay and occurred soon after prolonged rains, suggesting that waterlogging is an important factor. Furthermore, available phosphate and potash were relatively low. Unlike 'Gros Michel' in similar situations, diseased stools recovered and the new shoots produced fruit. A fifth and more serious outbreak also occurred on a badly drained soil after heavy rains and was undoubtedly aggravated by excessive application of ammonium sulphate (at least 2 lb. per plant within 4 months). The plants recovered after drainage had been improved, but more slowly than usual. A small plantation of 'Bluggoe' plantain, also normally resistant to wilt, suffered an attack under conditions of bad drainage and low soil phosphate and potash. In Natal (Wager, 1955), 'Cavendish' is widely grown and has proved resistant to wilt except, as far as is known, in one locality: here, in a small valley, the soil is heavy, poorly drained and often waterlogged. Under these conditions a large proportion of plants wilt and die, but others develop normally and bear fruit. In the Canary Islands, where 'Cavendish' is more frequently affected by wilt, the plants may encounter great variation in soil moisture content and in some instances are said to suffer from unbalanced fertilizing (Wardlaw, 1933). That short periods of flooding on well-drained soils do not markedly affect the resistance of 'Lacatan' to *Fusarium* wilt is indicated by the behaviour of plantations growing on light alluvial loam which had been flooded for several days. A few plants showed incipient wilting, apparently unconnected with fungal attack, and subsequent recovery was rapid and complete.

These field observations on the incidence of banana wilt were supplemented by pot experiments, to interpret which it is necessary to allow for the special conditions of glasshouse growth. Two findings reported earlier (Rishbeth, 1955) are particularly relevant, that rate of root formation markedly influences the number of rhizome infections and that rhizomes show little or no resistance to entry of the pathogen from infected roots. Small 'Gros Michel' plants were grown in pots of unsterilized light loam in sets of ten. Since the viability of *F. oxysporum f. cubense* in soil tends to decline, application of inoculum was usually delayed until 2–4 weeks after planting; and to avoid root damage, a standard sawdust/maize-meal inoculum was distributed over the soil surface, which was then lightly watered. During this and subsequent waterings, *Fusarium* spores were carried down into the soil. Rhizome infections, rising from invasion of roots predominantly through rootlets, were counted after 3 months. Table III shows the effect of various treatments on rhizome infection, expressed as the total number of separate infections occurring in ten inoculated plants. Control plants throughout were grown in earthenware pots, were watered every day for the first 5 weeks and thereafter on alternate days, and received no amendments. Because of variations in rate of root growth, inoculated plants subjected to a given treatment at different times of the year were often infected to varied extents.

TABLE III

The Number of Rhizome Infections occurring in Ten Inoculated 'Gros Michel' grown under Various Conditions in the Glasshouse

	Number of infections in	
Treatment	Control plants	Treated plants
Watering only once weekly	38	5^{+}
	19	2
Watering twice suspended	42	13^{++}
Waterlogged twice	42	33
Plants grown in baskets	27	70^{++}
Bagasse added to soil	38	1^{++}
	19	0^{+}
Nitrogen applied	42	106^{++}
Phosphate applied	51	78
Potash applied	51	65
Phosphate and potash applied	51	126^{+}

Significant difference from control plants:

$+P = 0{\cdot}05$
$++P = 0{\cdot}01$

With plants watered only once a week, root development was very slow and the low incidence of rhizome infection can be explained by a lack of infection courts. The intervention of two artificial 'drought' periods of a week each caused a similar reduction in rhizome infection. These results throw interesting light on the low incidence of wilt in dry weather. Two 24-hour periods of waterlogging had little effect on the incidence of infection; the result of waterlogging plants already infected will be described later. Bananas grown in baskets of split bamboo developed more infection than bananas grown in pots under otherwise comparable conditions, probably because roots were subject to a greater fluctuation of moisture in the former. By contrast, addition to the soil of 5 per cent. by weight of old bagasse (debris from the processing of sugar-cane) resulted in greatly reduced infection. This could not be attributed to delayed root development, as with restricted watering, since root growth was normal. Again, the nutrient effect of the amendment was negligible as was the microbiological effect, since the bagasse carried a very simple microflora and ability to reduce infection was retained after sterilizing. It seems likely that the smaller amount of infection is to be attributed to a reduced fluctuation in soil moisture content, associated with the high water-retaining capacity of the bagasse. In a small subsidiary experiment it was noted that a plant in a normally exposed pot developed 13 rhizome infections whereas two plants in similar pots embedded in moist peat developed only 2 and 4 infections respectively. These results support the conclusion, already suggested by field observations, that marked fluctuations in soil moisture predispose banana roots to infection, as Wardlaw (1930*a*) maintained.

The effect of heavy fertilizing with nitrogen was demonstrated by an experiment in which plants each received 1 g. ammonium sulphate twice weekly. The number of rhizome infections was greatly increased and individual infections were about three times as extensive as in plants not receiving nitrogen. Furthermore, in most plants *F. oxysporum f. cubense* grew sufficiently far to enter the pseudostem, where it grew to an average height of 13 cm., a rare occurrence in pot experiments. After 3 months the plants still showed no signs of wilt, however. The average number of mature roots per plant was the same as with unfertilized plants and hence the difference in infection between the two sets cannot be ascribed to differing rates of root development. The most likely explanation is that the pathogen grew faster in roots of the fertilized set, with the result that more root infections reached the rhizome.

In another experiment the effect of applying phosphate and potash to small 'Gros Michel' was tested. The former was applied as pure disodium hydrogen phosphate (50 per cent. P_2O_5) and the latter as commercial potassium sulphate (48 per cent. K_2O): with each, 0·45 g. was applied twice weekly. In plants receiving either phosphate or potash, rhizome infection was not greatly different from that in untreated plants, but in plants receiving both fertilizers it was significantly greater. In a parallel test with soil from two former factory sites, both very rich in available phosphate and potash, rhizome infection was similarly about twice as great as with the less fertile loam. The discrepancy between these results and the observed low incidence of wilt on soils naturally rich in these nutrients can perhaps be explained by postulating two distinct mechanisms of fertilizer action. Firstly, provision of phosphate and potash tends to promote root development and thus to increase the number of infection courts: root growth was in fact exceptionally good where both nutrients were added. Secondly, these nutrients, unlike nitrogen, do not lead to increased growth of the pathogen in vascular tissue and may, if taken up in sufficient quantity, bring about a significant increase in host resistance. In the experiments just described, it is believed that the first effect predominated and that uptake of nutrients during the relatively short growth period was insufficient to alter the low resistance to rhizome infection characteristic of potted plants. With plantations of 'Gros Michel' on soils naturally rich in phosphate and potash, however, the second effect apparently predominates.

iii. *Soil microflora*

With a soil-borne disease such as banana wilt, it is reasonable to consider the possible effect of microbiological factors. Attention will here be briefly directed to abundance of the pathogen, variations in its pathogenicity, and the part played by other components of the soil microflora.

There are in the literature relatively few references to the effect on banana wilt of the abundance of *F. oxysporum f. cubense* in soil. This is no doubt partly due to the fact that it is difficult to perform adequately controlled experiments demonstrating such an effect once wilt has become prevalent, and partly to the absence of satisfactory estimates of the pathogen's abundance. Moreover, the effect of soil population on wilt incidence is often masked by that of physical and chemical soil conditions. Apart from the classical experiment of Brandes (1919), perhaps the most striking demonstration of the effect of inoculating bananas with *F. oxysporum f. cubense* was provided by Drost (1912) in Surinam, before banana wilt had spread very widely there. Nine plots, each with 28 'Gros Michel', were inoculated with finely chopped naturally infected material, another set of nine plots being left uninoculated as control. Wilt soon appeared in the former plots, 29 per cent. of plants being affected after 9 months and 100 per cent. after 16 months. The latter plots still showed no disease after 15 months, although infection appeared later, especially in plots adjacent to inoculated ones. The number of fruit bunches reaped during the year after planting was 6 in the inoculated plots and 234 in the controls. A rather similar effect, appearing without deliberate inoculation, was seen at the Banana Breeding Station, Bodles, where part of the nursery used for planting out seedlings of varied susceptibility to wilt was formerly occupied by 'Gros Michel', some of them diseased. In the course of some 18 months, 50 plants developed wilt in this section whereas only 7 cases arose in an adjacent section of similar size not formerly planted with 'Gros Michel'. In these two instances, contrasted wilt incidence can be explained by differences in soil population of the pathogen.

In the current investigation, a field experiment was set up in an attempt to show the effect on wilt incidence of varying the amount of inoculum. The inocula employed were (1) soil taken from the vicinity of wilted plants and (2) small, medium, and large amounts respectively of finely chopped infected pseudostem, these inocula being applied to the soil around the bananas 10 weeks after planting. Each treatment was carried out in two plots of 20 'Gros Michel' each, plots being separated by two 'guard' rows; two similar uninoculated plots served as controls. Unfortunately the natural infective capacity of the soil was greater than anticipated and the lighter inoculations of chopped pseudostem gave similar results to the control.

Inoculation with soil (10 kg. per plant) and the heaviest inoculation with pseudostem (1·2 kg. per plant) produced after 6 months three and four times respectively the number of wilt cases appearing in control plots. Large inocula thus hasten appearance of the disease.

Supplementary observations were made on small 'Gros Michel' grown for 3 months under standard conditions in the glasshouse. The effect on rhizome infection of varying the dosage and mode of application of inoculum is illustrated in Table IV.

A banana produced on an average about 20 mature roots during an experiment and therefore the total number of rhizome infections possible in a series of ten plants is of the order of 200. Experiments 1, 2, and 4 show the effect of improving inoculum distribution, in that progressively smaller inocula caused roughly equivalent amounts of rhizome infection. The method used in experiment 1, though advantageous in avoiding root damage when performed some weeks after planting, was relatively inefficient in causing infection. In experiments 3–5, inoculum distribution possibly approximated to that found under natural conditions, and although some infection occurred with very low populations of *F. oxysporum f. cubense*, infection did not approach even half the theoretical maximum until the population was much greater (3,000/g.) than that believed to occur naturally. This suggests that in the field the pathogen seldom invades a high proportion of banana roots.

TABLE IV

The Number of Rhizome Infections occurring in Ten 'Gros Michel' Inoculated with Various Dosages of Fusarium oxysporum f. cubense *in the Glasshouse*

	Details of inoculum			
Experiment	Substrate	Mode of application to soil	Viable spores per g. soil	Number of infections
1	Sawdust/ maize-meal	Added to surface	10,000	19
2	,,	Mixed	700	14
3	Soil	,,	3,000	83
4	,,	,,	35	13
5	,,	,,	2	2

It seems likely that variations in soil population of the pathogen account for some of the apparently inexplicable differences in wilt incidence on adjacent sites where soil conditions are very similar, and for much of the erratic development of the disease throughout Jamaica in the past. In view of the relatively high soil population of *F. oxysporum f. cubense* occasionally found in Jamaican 'Lacatan' plantations, it is important not to ignore the possibility of a progressive build-up of the pathogen in soil, even with resistant varieties.

It must be admitted that little is known about variations in pathogenicity of *F. oxysporum f. cubense* and it is therefore well to realize that qualitative differences in the soil population of this organism may also affect wilt incidence. Since distinct physiologic races are known for other wilt pathogens such as *F. oxysporum f. lycopersici* (Gerdemann and Finley, 1951) and *F. oxysporum f. lini* (Houston and Knowles, 1953), it is worth discussing the evidence for their existence in the banana pathogen. Some workers believe, for instance, that the strain affecting 'Cavendish' in the Canary Islands is distinct (Wallace, 1952): however, this belief is apparently based on the assumption that 'Cavendish' is immune to wilt in the Caribbean area rather than on specific inoculation experiments. 'Cavendish' was one of the varieties tested in the experiment described earlier (p. 216) and was found to suffer very slight root infection with a Jamaican isolate of *F. oxysporum f. cubense* from 'Gros Michel', in this respect behaving like the related 'Lacatan'. Under experimental conditions of waterlogging (p. 238), however, the resistance of 'Cavendish' to this isolate was greatly reduced. This result supports the conclusion that such varieties are highly resistant rather than immune to Caribbean strains of the pathogen. Further, the circumstances of the Natal outbreak in 'Cavendish', together with those of the Jamaican outbreaks in 'Lacatan', strongly suggest that exceptional conditions, rather than a distinct race of the pathogen, are implicated.

In Jamaica, when the first serious outbreak of wilt occurred in 'Lacatan' and later when the new tetraploid variety '1877' became affected, it was suggested that new races of the pathogen had appeared. However, in view of the short interval between planting and appearance of wilt, only 7 months in the latter instance, it seemed unlikely that this was so. By means of a pot test known to give results closely according with field experience, it was established that isolates of *F. oxysporum f. cubense* from 'Lacatan' were not more pathogenic to this variety than an isolate from 'Gros Michel'. A comparison was also made between an isolate from the variety '1877' and another from 'Gros Michel', with a comparable result. There is thus no evidence that new races of the pathogen capable of attacking resistant banana varieties have appeared in Jamaica yet. On the other hand, it seems probable that differences in pathogenicity to a given variety are to be found within the existing race (Rishbeth, 1955).

Indirect evidence as to possible effects on wilt incidence of other elements of the soil microflora was derived from various experiments in the glasshouse. Comparisons were made between pairs of soils which were inoculated with comparable dosages of *F. oxysporum f. cubense* applied to the soil surface. Before inoculation the pathogen was not detectable in these soils, but their varying capacity to cause wilt in the field was later shown by experimental plantings of 'Gros Michel'. Pairs of inoculated soils usually gave very similar amounts of rhizome infection in 'Gros Michel', despite the fact that the original soils differed greatly with respect to microflora. An exception was provided by two very fertile soils from former sugar-factory sites which caused little wilt under field conditions but on inoculation in pots produced more rhizome infections than an infertile loam. A possible explanation for this has already been given (p. 223).

In other experiments a variety of soils, sterilized by autoclaving for 1½ hr. at a pressure of 20 lb. per sq. in., usually gave on inoculation amounts of rhizome infection similar to the corresponding unsterilized soils. However, roots tended to be fewer and somewhat stunted in autoclaved soils, possibly owing to the toxic effect of ammonia, and in one instance this led to a significantly lower incidence of rhizome infection. With a range of soils, therefore, sterilization did not lead to a higher incidence of infection despite its known tendency to promote a slower decline in viability of the inoculum. These observations suggest that the general soil microflora has little or no effect on initial root infection by *F. oxysporum f. cubense*, a conclusion supported by work described later (p. 234). In Trinidad, Rombouts (1953) similarly found no difference in the rhizosphere population or general microflora of two soils which could account for the contrasted wilt incidence in bananas growing on them. Further, there is no evidence that the type of microflora affects survival of the pathogen: indeed this would not be expected if it survives chiefly in the form of chlamydospores. This mode of survival seems likely in view of the longevity of these spores—at least 3 years—in dry soil cultures, and the observation by Warcup (1955) that in the Australian soil he examined *F. oxysporum* was present as chlamydospores.

It may be noted that bacterial wilt of bananas, caused by *Pseudomonas solonacearum* E. F. Smith, is absent or very rare in Jamaica, and thus it seems unlikely that the incidence of banana wilt is complicated by joint infection of bananas with the two pathogens. In Trinidad, by contrast, the two diseases were seen in adjacent 'Gros Michel' and there, as in the East Indies (Gäumann, 1921), the position may be more complicated.

iv. *Other factors*

Banana wilt often appears to be associated with a variety of other factors. Here, consideration will be confined to weather, often producing rapid and striking effects, and to crop management, generally causing gradual changes the effects of which are correspondingly difficult to establish. Such factors often operate indirectly, for instance by increasing the local population of the pathogen or by changing soil conditions.

Rainfall often has a marked effect on banana wilt. As Hansford (1926) states, there tends to be a noticeable reduction in the number of disease cases during drought and a sharp increase shortly after heavy rains. This is clearly shown by wilt incidence in 'Cavendish' in the Canary Islands (Wardlaw, 1933) and by that in experimental 'Gros Michel' plantations in Jamaica during the present investigation. In one such plantation 4 relatively dry months were characterized by a slow, steady appearance of wilt whereas 2 unusually wet months which followed were accompanied by greatly increased wilt incidence, three and four times the previous number of cases arising in successive 2-month periods. In another plantation wilt developed at a steady rate for 8 months after which there was a violent storm of wind and rain: here again wilt increased four-fold within 2 months. In each instance wilt increased sporadically and also in plants adjacent to existing cases: particularly with the latter, it appeared so rapidly that the plants must already have been infected. After high wind, a sharp drop in host resistance can probably be explained by severe mechanical damage to roots, but with heavy rain alone the reason is not clear. There is some evidence that 'Gros Michel' shows increased susceptibility to wilt after severe drought, as with the earliest recorded outbreak (Ashby, 1913). Rapidly growing bananas tend to wilt quickly whereas bananas checked in growth wilt slowly: thus in Jamaica wilting is far less common in hill country above 2,000 ft. during winter months, when night temperatures often fall below 60° F. and growth is correspondingly slow.

Considering the longer-term effects of rainfall in encouraging new infections, Brandes (1919) mentions the importance of profuse root growth, which provides new infection courts. It is further possible that heavy rains, often accompanied by local or more widespread flooding, play an important part not only in carrying the pathogen from place to place but in washing it down to the root system. Since there is reason to believe that spores of *F. oxysporum f. cubense* are important agents of root infection, a few experiments of the type described by Burges (1950) were carried out. The vertical soil column was held in a tube (Evans, 1955) which could be sampled through side-arms 5 cm. apart. Sterile tubes were filled with autoclaved soil and brought to the required moisture content by mixing thoroughly with sterile water. A small volume of a suitable suspension of *F. oxysporum f. cubense* microconidia was added to the soil surface, followed by a volume of sterile water equivalent to a depth of 2·5 cm. in the tube. About 2 hours later, soil samples were extracted with a sterile cork-borer and were plated at suitable dilutions with Dox's agar; any water draining from the base of the soil column was also plated. If the soil column had first been saturated by allowing sterile water to percolate through, spores added subsequently were washed down the full length of the column, 35 cm., when the measured volume was applied all at once. Under these conditions, spores were carried down more readily through loam than through clay, as might have been expected; in the case of loam it was remarkable that even with a small inoculum, giving a population near the surface of 120 spores per g. soil, spores were detectable in water draining from the column. On the other hand, spore penetration was more limited in loam at 40 per cent. moisture-holding capacity, when visible wetting occurred to a depth of only 20 cm. Again, penetration was much less when the measured volume of water was dropped on to saturated soil at a rate slow enough to prevent flooding. Some of the results obtained are reproduced graphically in Fig. 1. That the marked downward movement of spores was not entirely due to use of a narrow tube was indicated by a comparable experiment in a 12-in. pot. Here spores were introduced just beneath the soil surface at the centre only, and after the soil had been flooded to a depth of an inch, *F. oxysporum f. cubense* was detected almost as soon as water drained from the pot base. In tropical plantations, downward movement of spores in gravitational water doubtless occurs when the soil is flooded after irrigation as well as after heavy rainfall. Hickman and English (1951) have shown that transport of *Phytophthora fragariae* zoospores by soil-water movement is important in promoting infection of strawberry roots.

In view of the far-reaching effects on soil structure and fertility of growing bananas, it is not surprising that crop management, in the widest sense, has an important bearing on the incidence of banana wilt. At the outset, selection of site is crucial: Wardlaw (1935) records that in Central America considerable areas unsuitable for continuous cultivation of 'Gros Michel' were planted and that many sites were abandoned after a few years even in the absence of wilt.

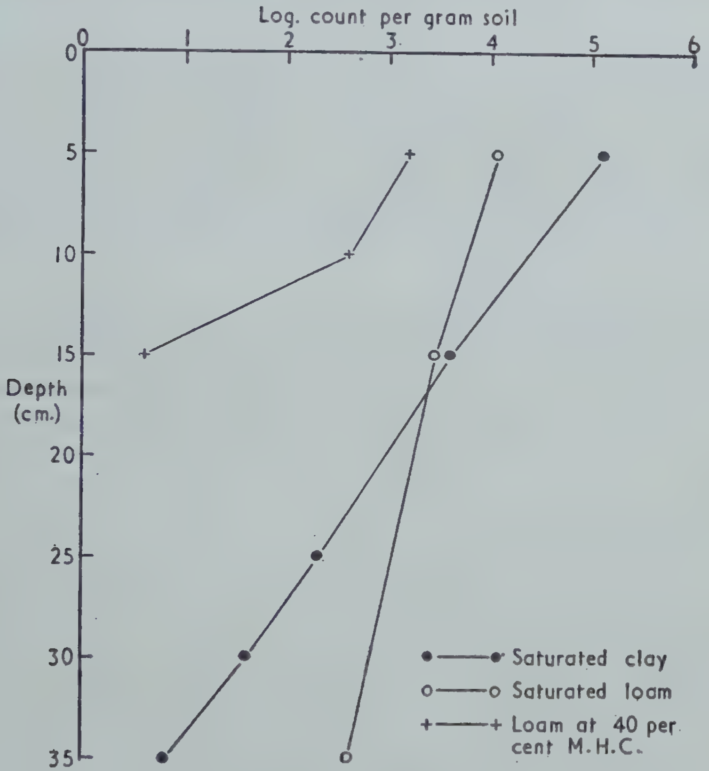

FIG. 1. Graphs showing downward movement of *F. oxysporum f. cubense* spores in gravitational water.

In other instances plantations produced poor crops and also suffered badly from wilt, these conditions being associated with various soil defects such as inadequate drainage or fertility. Hardy, Smart, and Rodriguez (1935), besides stressing the importance of soil structure and texture in relation to the water requirements of bananas, noted that the crop imposes a heavy demand on soil nutrients. They tentatively fixed the lower limits for available phosphate and potash, respectively, at 50 and 150 p.p.m. of soil for lighter soils and at 75 and 225 p.p.m. for heavier ones, under adequate conditions of soil moisture.

Jacob and Coyle (1931) quote data from Queensland that an average banana crop removed the following quantities of nutrients (lbs. per acre) from the soil: nitrogen, 84·5; phosphoric acid, 22·5; and potash, 271·5. These figures, if only approximately correct, indicate that the demand for potash is relatively very great.

Many of the 'Gros Michel' plantations included in a survey made during the present investigation (Table I) were on soils of much lower nutrient status than the limits quoted above, and some were on soils so impoverished that deterioration under bananas could hardly alone account for their condition: they must have been deficient initially. In this connexion it is of interest to record an observation made nearly 20 years earlier: Hardy and Croucher (1933) found that the soil of a Jamaican banana plantation in hill country, abandoned through wilt, had only 6 p.p.m. phosphate and 80 p.p.m. potash. These observations suggest that in Jamaica, as in Central America, many soils planted with bananas were too infertile to allow reasonable growth or yields of fruit; they also help to explain why wilt spread so rapidly in some plantations when *F. oxysporum f. cubense* eventually reached them. It is noteworthy that several such sites have been included in the widespread replanting with 'Lacatan'.

In Jamaican banana cultivation, two practices in particular have tended to affect soil structure and fertility, lack of rotation and 'clean-weeding'. The former is influenced mainly by short-term economic considerations, bananas being not only a valuable crop but yielding a relatively steady financial return. Only locally, as in the Buff Bay valley, has there been any deliberate departure from continuous cultivation. The second practice derives from the belief that satisfactory fruit quality can only be attained if plantations are kept free from weeds. In many banana-growing areas, for instance, rainfall is considered insufficient to prevent serious competition for water between bananas and other plants. Earlier, in the general absence of fertilizing and other measures of soil conservation, these practices led to varying degrees of soil deterioration. In many instances, as with rendzina soils overlying stiff clays and with soils derived from purple shales and sandstones, erosion was widespread and severe (Leach, 1946). This erosion caused a marked increase in susceptibility of 'Gros Michel' both to wilt and leaf-spot (*Mycosphaerella musicola*, Leach). In the survey described earlier, distribution of wilt often corresponded closely with that of erosion: in one instance the only patch of disease in an otherwise healthy crop was located at a site having no topsoil.

Apart from such spectacular effects, there is little doubt that prevailing cultural practices often led to a decline in soil fertility. Evidence for this is available from observations on the potash status of irrigated soils. In a newly planted field of 'Gros Michel' at the Banana Breeding Station, Bodles, available potash in four unfertilized plots diminished by 130–250 p.p.m. (average 180 p.p.m.) in one year. The bulk of this loss was presumably due to uptake of potash by the bananas, but on very light soils there is probably some loss by leaching. Thus in a sandy loam, fallow at the time, available potash increased from 150 to 300 p.p.m. soon after adding potassium chloride or sulphate but declined to the original value after only 3 months, during which period the site was irrigated three times and then received heavy rainfall. Here the base-exchange capacity was undoubtedly low. There is also circumstantial evidence that soil fertility declines, though more slowly, under bananas in non-irrigated sites. Thus at an old factory site where 'Gros Michel' had grown continuously for 40 years and was now rapidly going down with wilt, the soil was relatively low in available phosphate (26 p.p.m.) and potash (170 p.p.m.): by comparison with many similar sites, the soil must originally have been far more fertile. Until recently, balanced fertilizing was scarcely practised and bananas were either not fertilized or received nitrogen only. Formerly, nitrogen was often given to plantations declining in vigour, which resulted in a temporary improvement in growth, though not always in fruit production. Later, however, deterioration was usually rapid, particularly through wilt, which was doubtless aggravated by the unbalanced fertilizing.

Some planters believe that cultivation, especially mechanized cultivation as practised by larger estates, affects the incidence of banana wilt. In one alluvial area the earlier lengthy survival of 'Gros Michel' was attributed to less intensive cultivation and in particular to the method of replanting, where stools were removed and bananas planted in the same position; cultivation was carried out only between rows of plants. Later practice involved thorough deep cultivation of the whole site before replanting and although yields of fruit were at first much better, crippling attacks of wilt invariably developed within a few years. This outcome is probably attributable to a more widespread dispersal of *F. oxysporum f. cubense* in the soil. To a lesser extent, frequency of forking is thought to be connected with wilt incidence, and in support of this it was noted that old healthy plantations of 'Gros Michel' were forked only occasionally, at intervals varying from 6 months to several years.

With some soils, provision of adequate drainage ditches is essential for good banana growth, and an instance has been quoted (p. 221) where recovery of 'Lacatan' from wilt followed improved drainage. However, there is almost certainly an optimum depth for such ditches, as Drost (1912) pointed out, and failure to recognize this may have contributed to outbreaks of wilt in two old plantations where ditches up to 1·5 m. deep probably caused undue fluctuations of soil moisture. The question of hygiene within plantations will be discussed more fully elsewhere, but it may be noted here that instances still come to light illustrating the part played by cultural practices in disseminating the pathogen: the importance of banana trash and contaminated tools has often been mentioned before.

v. *Interaction of factors*

Factors affecting banana wilt interact to a bewildering extent. An example of such interaction under Jamaican conditions is given in Table V, which for three sites records wilt incidence in banana varieties of differing resistance to the disease. Approximate estimates of the population of *F. oxysporum f. cubense* were obtained by the method described elsewhere (Rishbeth, 1955).

A tendency for wilt to occur sporadically on light soils heavily infested with *F. oxysporum f. cubense* distinguishes '1877' and some other new tetraploid varieties from 'Lacatan'. Appearance of wilt in 'Lacatan' on soil (1), which was badly drained, is probably to be ascribed to temporary waterlogging. The fact that this variety succumbed to wilt on soil (1) with a low population of the pathogen but not on soil (2) with a high one strongly suggests that major factors here were the physical and chemical properties (including pH) of the soil.

TABLE V

Wilt Incidence in Certain Banana Varieties in Relation to Conditions existing in Three Jamaican Soils

Soil conditions				Wilt incidence in		
Texture	pH	Available nutrients	Population of the pathogen	'Gros Michel' (susceptible)	'1877' (resistant)	'Lacatan' (very resistant)
(1) Stiff clay	5·4	Medium P High K	Low	+	(+)	(+)
(2) Sandy loam	8·0	Medium P Low K	Very high	+	(+)	—
(3) Medium loam	7·0	High P High K	Low	(+)	—	—

Wilt incidence: + heavy; (+) light; — none.

On the other hand, there are indications that a slow build-up in soil population of the pathogen may eventually offset the benefit, with respect to wilt resistance, of high soil nutrient status. Thus a number of 'Gros Michel' plantations on sites of former sugar factories, subject to frequent flooding, have recently suffered a gradual encroachment by wilt despite having highly fertile soils. It is further possible to argue that, owing to the greater prevalence of local flooding after heavy rain and to more frequent general flooding from rivers and streams, *F. oxysporum f. cubense* is now more abundant and widely dispersed in lowland soils than in upland ones, where in addition the relative isolation of banana plantations would often tend to delay its dispersal. This could explain the tendency for lowland soils bearing old healthy 'Gros Michel' plantations to have more available phosphate and potash than upland ones (Table I), conditions for wilt resistance having become more exacting with the former. However, in the absence of more sensitive methods for estimating abundance of the pathogen in soils, it is impossible to prove such a hypothesis. In general it may be concluded that wherever the pathogen is present, no single soil factor such as alkalinity, heavy texture, or high nutrient status is sufficient to confer wilt resistance on 'Gros Michel': a combination of such factors is essential, and therefore much good agricultural land is now unable to support a healthy crop of this variety.

WILT RESISTANCE

i. *Location of defence mechanisms*

Before discussing just how external factors affect wilt incidence, it is necessary to consider the various points at which *F. oxysporum f. cubense* is checked in bananas and to describe natural and experimental conditions under which resistance at these points may be modified. In so doing, it is relevant to examine the possible basis for varied wilt resistance in different banana varieties. Part of the answer to this intractable problem is to be sought in a study of the earliest stages of infection in varieties of contrasted wilt resistance. Plants of the resistant 'Cavendish' and highly susceptible 'Gros Michel' were grown under comparable conditions in pots of unsterilized loam/sand (1:1), and about 6 weeks after planting, when root growth was vigorous, the soil surface was inoculated with a concentrated spore suspension of *F. oxysporum f. cubense*. Portions of root in the surface soil were examined at intervals. One week after inoculation many early stages of infection could been seen on rootlets of each variety; roots of an uninoculated but otherwise comparable 'Gros Michel' were healthy. The presence of the pathogen in lesions was demonstrated for each variety by plating washed rootlets on acidified plain agar. Infections were characterized by a bluish-purple discoloration, particularly evident in cell walls: in 'Cavendish' they were always small at this stage whereas in 'Gros Michel' they were sometimes extensive and often characterized by a marked disorganization of cell contents.

After 2 weeks, some 50 per cent. of 'Gros Michel' rootlets were heavily infected, with purplish lesions more or less coalescing along the whole length, whereas in 'Cavendish' rootlet infection was usually less than 10 per cent. and lesions still tended to be localized. Plating showed again a profuse growth of *F. oxysporum f. cubense* from lesions and, with some 'Gros Michel' rootlets, growth from the vascular strand at the rootlet base. A study of rootlets cleared in lacto-phenol, compressed between microscope slides and mounted in dilute cotton blue indicated that infection occurred at any position along the rootlet. It was difficult to see *Fusarium* hyphae in the tissues: however, the general impression gained was that, although infection tended to spread inwards towards the vascular strand, the latter was not invariably penetrated in 'Gros Michel' and was infrequently penetrated in 'Cavendish'. Finally, after 3 weeks, 'Gros Michel' had a very high incidence of rootlet deaths (often nearly 100 per cent.) although a few new rootlets had been produced. Rootlet bases were often discoloured and vessels in the main root showed early stages of infection. The incidence of rootlet death in 'Cavendish' was lower (often about 20 per cent.) and here too a few vessels in the main root were infected. By this time, affected rootlets on plating produced a high proportion of secondary organisms such as *Trichoderma viride* Pers. ex Fr. and *Fusarium solani* (Mart.) Appel and Wollenw. emend. Snyder and Hansen. Another comparison was made in glasshouse experiments of 3 months' duration, this time between 'Gros Michel' and 'Lacatan'. With the former variety, two series of 10 plants potted in unsterilized light loam and inoculated with *F. oxysporum f. cubense* showed vascular discoloration in 14 and 22 per cent. of roots respectively whereas two comparable series of the latter variety had discoloration in only 3 and 4 per cent. respectively.

Several tentative conclusions may be drawn from these observations. Firstly, since the earliest infections appear very soon after inoculation, are widely distributed over the root system, and each involve only a few cells at the rootlet surface, it seems certain that the infective units must be spores, washed down through the soil by watering. And since infection commonly progresses a certain distance in 'Cavendish', toxic root secretions cannot play a major part in controlling infection at the root surface as they appear to do, for instance, in some wilt-resistant flax varieties (Timonin, 1941). However, the difference between the two situations may be one of degree only. The effect of excretions from living roots on germination of *F. oxysporum f. cubense* spores was studied in a preliminary way, but no difference in this respect could be detected between 'Cavendish' and 'Gros Michel'. A claim by Harper (1950*b*) that wilt resistance in banana varieties is related to the nature of the rhizosphere population was later refuted by the more extensive work of Rombouts (1953), who was unable to find evidence of any preferential selection of antagonists to the wilt pathogen in the rhizospheres of resistant varieties. Under the particular conditions of the inoculation experiments just described, no evidence was found of microbial competition at the earliest stages of rootlet infection, although competition sometimes developed later between *F. oxysporum f. cubense* and *Rhizoctonia solani* Kühn, which caused independent infections.

It may be noted in passing that the wilt pathogen is less sensitive to competition with *Trichoderma viride* than some other fungi. Of 12 *T. viride* isolates obtained from soil or banana roots and tested for interaction with *F. oxysporum f. cubense* on potato dextrose agar at 28° C., 6 were overgrown, 2 partially overgrew the *Fusarium* and 4 completely overgrew it. *Fusarium* spores often remained viable after such overgrowth by *T. viride* and in other experiments it was shown that *Fusarium* spores germinated in only slightly diluted extracts of gliotoxin and viridin and moreover remained viable after prolonged exposure to concentrated extracts of these substances.

F. oxysporum f. cubense might grow more slowly in rootlets of 'Cavendish' than in those of 'Gros Michel' because of a more effective reaction by the host tissues, or because some toxic substance is present. The former explanation appears more probable since Harper (1950*a*) was unable to find any stable substance in roots or rhizomes of a resistant variety preventing growth of the wilt pathogen whereas Wardlaw (1930*b*) demonstrated the importance of various tissue reactions in checking its growth. It is further just possible that *F. oxysporum f. cubense*, once established in vascular tissue, is unable to grow as freely in 'Cavendish' as in 'Gros Michel' because some important nutrient in the tracheal fluid is limiting. In view of the meagre nutrient requirements of the pathogen (p. 239), any absolute deficiency is most unlikely. Whatever the precise mechanism of this resistance, the ultimate effect is clear: at corresponding intervals after inoculation, infection in 'Cavendish' and 'Lacatan' is always less pronounced than in 'Gros Michel'. And although with the heavy inoculum used in the foregoing experiments the resistant varieties suffered some infection of main roots, it is possible that under favourable field conditions infection is often confined to rootlets. If, however, infection does proceed farther, other defensive mechanisms of the type described by Wardlaw (1930*b*) may operate: thus vascular infections of various origins are often blocked at the root base. Of 12 *F. oxysporum* isolates obtained from vascular tissue at this position and tested for pathogenicity (Rishbeth, 1955), 1 out of 4 from 'Gros Michel' and none out of 8 from 'Lacatan' proved pathogenic. Thus with the former variety at least we have direct evidence that the pathogen is blocked at this stage.

Further information about the ability of *F. oxysporum f. cubense* to penetrate root bases was obtained from inoculation experiments at a number of sites, selected for contrasted soil type and wilt incidence. Twenty young roots of 2-year-old bananas (about 5 per plant) were partially exposed at a region of good rootlet development and inoculated by covering with 100 g. soil containing some 1×10^8 viable spores of *F. oxysporum f. cubense*. Roots were ringed with adhesive tape and further marked by a peg to facilitate later recovery, were covered with soil and then watered. Roots which were not inoculated but otherwise received similar treatment served as controls. After 9–10 weeks, roots were exposed to their origin from the rhizome and the bases of all roots showing vascular discoloration at this point were further marked with tape. Rhizomes were then excavated and cut open to determine how many vascular infections had entered. With 'Gros Michel', 1 rhizome infection had developed from 18 infected root bases at one site where bananas had not previously been grown, and 1 from 19 such root bases at each of two sites where wilt was already prevalent. No rhizome infections were recorded from uninoculated roots, in which the incidence of vascular infection was usually much lower. Thus under varied field conditions 'Gros Michel' exhibits a certain amount of resistance to rhizome infection.

It was clearly difficult with such a low incidence of rhizome infection to obtain satisfactory comparisons but it may be recorded that at the first site 'Lacatan' developed no rhizome infections from 15 infected root bases. At this site 'Gros Michel' heavily fertilized with nitrogen (4 oz. ammonium sulphate per plant, monthly) not only developed 4 rhizome infections from 18 infected root bases, as compared with 1 in comparable unfertilized plants, but suffered considerable root infection from an inoculum that was relatively ineffective with such plants. The more extensive growth of *F. oxysporum f. cubense* in bananas receiving nitrogen, already noted in glasshouse experiments, is thus correlated in the field with a partial breakdown of resistance at root bases. When 'Lacatan' was heavily fertilized with ammonium sulphate in the glasshouse or in field experiments on a variety of soil types there was no increase in rhizome infection. On the other hand, the severe outbreak of wilt in 'Lacatan' described earlier (p. 221) indicates that once resistance to *F. oxysporum f. cubense* has been broken down by unduly adverse conditions, heavy nitrogen fertilizing has an effect on the pathogen's growth similar to that in 'Gros Michel'. It may be conjectured that such fertilizing does not appreciably affect the issue of the first vital host reaction in rootlets but does encourage more vigorous growth of the pathogen once it has become established in vascular tissue. This latter suggestion is in accordance with observations on other vascular pathogens such as *Verticillium albo-atrum* Reinke and Berth. (Roberts, 1943) and *Xanthomonas stewarti* (E. F. Smith) Dowson (McNew and Spencer, 1939). Circumstantial evidence, based on field experience, that older 'Gros Michel' show increased resistance to wilt has been given elsewhere (Rishbeth, 1955). In the root inoculation experiment just described, a comparison was made at one site between 2-year-old and 20-year-old plants, roots being examined for vascular discoloration which was scored 0–3 on the basis of increasing severity. The mean score for young plants, 1·6, differed significantly from that for old plants, 0·9, thus confirming the impression that under some circumstances the root system of older plants is more resistant to infection by *F. oxysporum f. cubense*.

The field observation that only a proportion of root infections penetrate the rhizome helps to explain how the abundance of *F. oxysporum f. cubense* in soil is related to wilt incidence. At one extreme, the soil population may be so small that susceptible varieties remain healthy under varied conditions: this was probably the case wherever 'Gros Michel' was grown for the first time on a large scale and may still be the case, for instance, in parts of Colombia, where wilt is very rare. With a slightly higher population of the pathogen, wilt tends to develop on poor soils but not on very fertile ones. As the population rises still higher, more roots are infected and the chances of the pathogen entering the rhizome are thereby increased. Possibly with very severe infections an additional factor is the incapacitation of part of the root system, although here the varying extent to which root death occurs from other causes must be taken into account.

On soils of low to medium fertility, recovery of 'Gros Michel' from wilt is rare and, at the best, only temporary; on exceptionally fertile soils, however, recovery is common, an affected stool often producing good fruit for many years. Interesting intermediate conditions may occur on soils of moderately high fertility: at one such site some shoots usually wilted in the wet season but the affected stools often recovered in the dry one. Another 'Gros Michel' plantation was under observation for 3 years, during which period wilt incidence varied considerably. Some 2 years after planting, 20 per cent. of the plants were affected by wilt and 18 months later wilt incidence had increased to 25 per cent., but nearly half the plants recorded before as being diseased showed no symptoms. Only 3 months later, wilt incidence had dropped to 16 per cent., and already half the recently affected plants appeared to have recovered. Shortly afterwards the crop was blown down by a hurricane, with the result that 10 months later over 40 per cent.

plants were affected and only a fifth of those showing symptoms a year before had recovered. Evidently under such conditions the balance between host and parasite is delicately poised. By contrast with 'Gros Michel', recovery of 'Lacatan' from wilt is generally complete.

An ability to check rhizome infection was also shown by 'Gros Michel' infected in the glasshouse and transplanted after 3 months to a site where bananas had not previously been grown. Many produced good fruit and were still healthy a year later. It was discovered that infection in the original small rhizome had become stabilized and that the newly formed rhizome above it became critically infected only if its own roots were invaded. Leoncio (1930) describes a very similar recovery of *Musa textilis* Née on transference from pots to the field. This observation prompted a field experiment to compare wilt incidence in 'Gros Michel' developing from healthy and clearly infected suckers of the larger size used in normal planting practice. The experiment was laid down at three sites, at one of which however conditions proved unfavourable and led to erratic growth; in the other two growth was excellent. Some details of soil type and the number of plants which had wilted after a year is shown in Table VI.

TABLE VI

Wilt Incidence in 'Gros Michel' grown from Healthy and Diseased Suckers on Two Jamaican Soils

Soil conditions		Plants grown from			
		Healthy suckers		Diseased suckers	
Texture	Available nutrients	Total no.	No. wilted	Total no.	No. wilted
Sandy loam	Medium P Low K	27	9	27	19
Heavy loam	Very high P Very high K	20	0	26	7

At each site plants developing from infected suckers were more liable to wilt than those from healthy suckers, as might have been expected, although with the former wilt did not follow so rapidly as some earlier observers have indicated. Apparently there was a greater tendency at the more fertile site for infection in the planting bits to become stabilized, much as, at similar sites, infected rhizomes often check the pathogen and produce healthy shoots.

Finally, some small-scale experiments were performed on infected plants in the glasshouse to determine the effect of altering the balance between shoot and roots. Usually in pot experiments with 'Gros Michel' a considerable number of root infections reach the rhizome, but soon after entry they are checked; with resistant varieties, infection is less pronounced and generally confined to the roots. One small 'Gros Michel' developing from a severely rotted rhizome developed wilt symptoms 5 weeks after inoculation of the soil with *F. oxysporum f. cubense*, the first time this had occurred in pot experiments. On examination, the plant was found to depend on a relatively small root system developed from the base of the pseudostem and not from the rhizome, as was normally the case: as with the transplanted 'Gros Michel' recently described (p. 237), infection through this root system had proved critical. It was thought that the plant might have wilted because the root system was smaller than usual and to test this hypothesis some vigorous 'Gros Michel' with early stages of rhizome infection were subjected to various degrees of root and rhizome pruning and were then replanted. Strongly pruned plants partially wilted after 1 week but subsequently recovered and remained healthy for 3 months, when examination revealed that root growth had been very vigorous and infection in the rhizome had been checked.

TABLE VII

Results obtained by Waterlogging Infected 'Cavendish' in Pots

	Soil type	Period of water-logging (days)	Effect on roots	Subsequent root growth	Incidence of rhizome infection
Experiment (1)					
	Heavy clay	6	All killed	Poor	Heavy
	,,	0	,,	,,	,,
	Loam/sand	6	,,	Vigorous	Slight
	,,	0	None	,,	None
Experiment (2)					
	Clay	3	All killed	Vigorous	Slight
	,,	0	A few killed	,,	None
	Loam/sand	3	All killed	,,	,,
	,,	0	A few killed	,,	,,

Unfortunately it was not possible to investigate this situation farther, but from experiments with 'Cavendish' described below, it is probable that recovery, which occurred even after removal of most of the rhizome, was attributable to profuse root growth resulting from regular watering.

It seemed likely that the occasional breakdown in wilt resistance of 'Lacatan' and 'Cavendish' after waterlogging in clay soils was largely due to root damage. To investigate this possibility two well-rooted 'Cavendish' were transferred to pots of loam/sand and two to pots of very heavy clay, which was then well compacted. The pots had previously been coated on the inside with bitumastic paint, to reduce aeration. After 10 days the soil was inoculated with *F. oxysporum f. cubense* and after 3 weeks one pot of each soil type was stood for 6 days in water, the level of which reached almost to the soil surface. A second and similar experiment was performed but with unpainted pots, less heavy and uncompacted clay, and a reduced period of waterlogging (3 days). The results of examining roots and rhizomes, in each case a month after waterlogging, are given in Table VII.

In experiment (1) aeration was evidently so poor in the heavy clay that roots died even without waterlogging. Although this treatment also killed roots in loam/sand, subsequent root growth was vigorous enough to promote some recovery of resistance to the pathogen, by contrast with the situation in heavy clay. In experiment (2) a few roots were killed by transplanting alone. Conditions favoured renewed root growth better than in the previous experiment and there was a higher resistance to rhizome infection. In the field the result of temporary waterlogging may thus depend not only on the extent of damage to the root system but on the rate at which it recovers. This probably accounts for the different behaviour of 'Lacatan' (p. 221) on light loam and heavy clay, both of which were known to contain *F. oxysporum f. cubense.*

ii. *Possible mode of action of external factors*

Any explanation of the ways in which external factors affect wilt incidence must necessarily be very speculative. Two general points may be raised: firstly, the banana, though grown under widely varied conditions, is in origin a forest plant and may, for instance, be ill adapted to deal with undue fluctuations in soil moisture or with poor soil aeration. Although our ignorance of banana physiology is almost complete, field experience suggests that this is so: growth of 'Gros Michel' is most responsive to soil conditions and a remarkable acceleration of growth-rate sometimes follows improved drainage (Leach, 1946). Secondly, *F. oxysporum f. cubense* is almost from the outset confined to the vessels, where the scanty mycelium suggests growth under conditions of near-starvation. The nutrient requirements of the pathogen, judging by experience with three isolates, are exceedingly simple, growth occurring in a basal medium of salts with a wide range of compounds as the sole source of carbon. One of these isolates was further used to determine the effect on mycelial growth in very dilute solutions of altering the supply of carbon and nitrogen. 20 ml. lots of a solution containing 0·001 per cent. potassium di-hydrogen phosphate and 0·0002 per cent. each of potassium chloride and magnesium sulphate were transferred to culture bottles. Glucose was then added to give two series of 0·008 and 0·08 per cent. carbon respectively. To each series, nitrogen was added either as sodium nitrate, giving 0·0004, 0·002, and 0·01 per cent. nitrogen respectively, or as ammonium sulphate, giving 0·002 and 0·01 per cent. nitrogen respectively. Three replicate bottles of each concentration were inoculated with about 100 spores, obtained from a culture on plain agar. Bottles were incubated at 25° C. for 2 weeks after which mycelia were washed, dried, and weighed. Mycelial weights, expressed in mg. per 20 ml. culture solution, are recorded in Table VIII.

At the lower glucose concentration growth was feeble throughout, though at comparable nitrogen concentrations it was rather better with the ammonium salt than with the nitrate. This is interesting in view of the fact that banana wilt is sometimes more severe in plants fertilized with ammonium sulphate than in those receiving sodium nitrate (p. 221). At the higher glucose concentration growth was far more profuse except at the lowest concentration of sodium nitrate, where nitrogen was clearly limiting. At certain critical levels, therefore, relatively small changes in concentration of carbon and nitrogen markedly affect the growth of *F. oxysporum f. cubense.* It follows that the influence of external factors on the host-parasite relationship may often be explained by their effect on the composition of the tracheal fluid, as Shear and Wingard (1944) maintain.

For wilt to develop, the pathogen must have grown to some extent in the rhizome and usually in the pseudostem also, although wilting commonly occurs in advance of mycelial growth and may well be associated with the production by the fungus of pectic enzymes, as shown for tomato wilt by Winstead and Walker (1954). Whereas a plant is unlikely to wilt in less than 2 months from the time of initial infection, a plant already infected but showing no symptoms may wilt within a few days of some marked change in external conditions. This suggests that under some circumstances the pathogen may spread rapidly in the rhizome. Young, fast-growing 'Gros Michel' are particularly susceptible to wilt, possibly owing to a relatively poorly developed root system. At this stage any extra demand made on an infected plant's reserves may adversely influence the balance between host and parasite, and this suggestion is supported by the marked tendency for wilt to develop at the time of fruiting. By contrast, any check to banana growth, for example, through low temperature, often affects this balance in favour of the host, if only for a time. Latterly, the chief problem of 'Gros Michel' cultivation in Jamaica has been the great difficulty of bringing a young crop through this early stage without a crippling attack of wilt, even where it followed a former healthy crop. A slow build-up of the pathogen as well as a gradual decline in soil fertility doubtless often contributed to this situation, brought to a head by the substitution of young, susceptible plants for old, resistant ones. Once wilt appeared, the pathogen sharply increased in numbers and the crop often declined rapidly.

TABLE VIII

The Effect on Growth of Fusarium oxysporum f. cubense *in Dilute Solutions of altering the Supply of Carbon and Nitrogen*

		Mycelial weights (mg./20 ml.) with Nitrogen source				
		Sodium nitrate			Ammonium sulphate	
	%N. %C.	0·0004	0·002	0·01	0·002	0·01
Carbon source glucose	0·008	0·7	0·8	0·5	1·8	1·2
	0·08	1·7	10·4	9·4	11·1	10·3

Site factors perhaps operate predominantly through their effect on the host, though they may also affect the pathogen. Thus soil texture is probably of major importance in affecting aeration and the water régime but has also been shown to affect the ability of *F. oxysporum f. cubense* to form spores (Stover, 1956): sporing is more profuse in sandy loam than in clay loam. The effect of soil texture on the downward movement of spores in gravitational water has already been noted. It is probably significant that old healthy

'Gros Michel' plantations are only to be found where the surface soil is well aerated: with bad aeration the root system is probably poorly developed and may also be less efficient in taking up important nutrients such as potash (Hammond, Allaway, and Loomis, 1955). Bananas on sandy soils low in organic matter may be susceptible to wilt not only because of marked fluctuations in moisture content but because potassium is freely mobile in them (Russell, 1950).

Uptake of nutrients from the soil is clearly most important. It has already been suggested that nitrogen fertilizing tends to increase wilt mainly through allowing more extensive growth of the pathogen in vascular tissue. It is not clear why high potash status of the soil is correlated with wilt resistance, but Shear and Wingard (1944) quote evidence that with some plants a relative deficiency of potassium leads to increased nitrate in conductive tissue and suggest that this may promote growth of vascular pathogens. Bananas apparently demand so much potash that deficiencies are often likely to arise. Phosphate generally has an indirect effect on root growth by promoting leaf development and carbohydrate production, and this may partly account for the fact that healthy 'Gros Michel' plantations tend to be found on phosphate-rich soils. In glasshouse experiments, addition of phosphate and potash stimulated root growth without increasing resistance to infection, for the possible reason discussed earlier. By contrast, 'Gros Michel' growing on soils very rich in such nutrients have great vigour and considerable resistance to wilt: the disease rarely spreads from one plant to another and affected plants usually recover. Under these conditions the soil population of *F. oxysporum f. cubense* probably remains low: the pathogen was in fact not detected in 3 very old plantations by a glasshouse test (Rishbeth, 1955). With the combination of high soil fertility and low *F. oxysporum f. cubense* population, it is understandable that 'Gros Michel' remains healthy for many years unless subject to frequent flooding, which doubtless weakens the plants as well as introducing fresh inoculum. Although on some less fertile Jamaican soils 'Gros Michel' responds to potash fertilizing by increased fruit yield, apparently insufficient potash is taken up materially to affect wilt incidence. The data in Table I suggest that, to achieve wilt resistance, this variety must take up far more potash than is necessary for normal growth and fruit production, at least on alluvial soils.

Soil reaction probably operates for the most part through nutrient uptake. Thus under acid conditions many plants absorb considerable amounts of manganese and aluminium and accumulation of the latter in roots may interfere with translocation of phosphate, even when this is abundant (Russell, 1950). Alkaline conditions may be favourable mainly because soil micro-organisms are more active, leading to a relatively rapid release of plant nutrients from organic matter.

The effects of weather on wilt incidence are amongst the most interesting. The breakdown of wilt resistance in 'Lacatan' on some clays after heavy rain has already been discussed. It seems likely that less drastic conditions are sufficient to upset the balance between host and parasite in 'Gros Michel', which commonly wilts after heavy rain on loam as well as on clay. Field evidence suggests that although few 'Gros Michel' wilt during dry weather, a period of drought predisposes plants to wilt later, when rains follow: a similar effect has been established experimentally by Foster and Walker (1947) for *Fusarium* wilt of tomatoes. It is not clear whether marked fluctuations of soil moisture encourage root infection through creating improved nutrient conditions for the pathogen in the host, or more directly by lowering host resistance. Bananas are ill adapted to withstand high wind and much damage to root systems must occur short of actual wind-throw: the resultant effect on wilt incidence has already been mentioned. The part played by a vigorous root system in checking growth of the pathogen, clearly seen when it is impaired by mechanical damage, is a striking feature of banana wilt. Wilt resistance may in fact largely depend on the ability of a banana to develop and maintain an adequate root system, and in this connexion the relative resistance to wilt of old 'Gros Michel' and of plants growing on soils rich in phosphate and potash is probably significant. Such considerations should not be ignored in attempts to control the disease: thus the fact that with some plants concentrations of β-indolylacetic acid as low as 1 part in 10^{11} stimulate root growth, though insufficient to affect shoots (Audus, 1955), may possibly prove useful.

SUMMARY

Some banana varieties such as 'Lacatan' are highly resistant to wilt: such resistance, determined genetically, may be demonstrated by a glasshouse test.

In Jamaica, where infection is now widespread, old healthy plantations of 'Gros Michel' are virtually confined to alkaline soils of medium to heavy texture which are well drained and aerated, rich in available potash and not subject to frequent flooding. Severe attacks of wilt develop in 'Gros Michel' under a wide range of soil conditions, such as acidity, light texture, low nutrient status and poor drainage. Nitrogen fertilizing tends to increase susceptibility to wilt. On clay soils, resistant varieties occasionally develop wilt after heavy rains, probably owing to temporary waterlogging.

Glasshouse experiments are described which illustrate the influence on rhizome infection of different treatments given to small 'Gros Michel'.

Variation in the abundance of *F. oxysporum f. cubense* in soil affects wilt incidence. Little is known of variation in pathogenicity of the organism, but distinct races do not appear to have arisen yet. The general soil microflora has little influence on root infection by the pathogen.

Weather often produces striking effects: thus dry weather is generally accompanied by a temporary decrease in wilt incidence and heavy rainfall by an increase. One probable effect of heavy rainfall is to wash spores of *F. oxysporum f. cubense* from the soil surface down to the root system.

Prevailing practices in banana growing have often led to soil deterioration, varying from severe erosion to a slow decline in fertility, and thus to increased

wilt incidence. Other practices, such as mechanical cultivation, have probably increased local distribution of the pathogen.

Observations on early stages of rootlet infection indicate that the pathogen grows more slowly in the resistant 'Cavendish' than in the susceptible 'Gros Michel', probably because tissue reactions are more effective in the former.

Under varied field conditions, 'Gros Michel' can seal off infections at root bases, the pathogen being found to enter the rhizome from only 1 out of some 20 infected roots. Heavy fertilizing with nitrogen causes a partial breakdown of resistance at this point. Roots of old plants tend to be more resistant to infection than roots of young ones.

Resistant varieties usually recover from wilt rapidly; by contrast, 'Gros Michel' seldom recovers except on soils of high nutrient status, where infection often becomes stabilized in the rhizome. This also occurs in small 'Gros Michel' infected in the glasshouse and then transferred to the field: such plants seldom wilt unless subsequently infected through new roots.

In the glasshouse, infected 'Cavendish' were subjected to temporary waterlogging which killed roots and lowered resistance to rhizome infection. Recovery of resistance was apparently related to the rapidity of new root growth.

The possible mode of action of various factors on wilt incidence is discussed. It is thought that factors often operate through their effect on composition of the tracheal fluid: at certain levels, small changes in concentration of carbon and nitrogen were shown markedly to affect growth of *F. oxysporum f. cubense* in very dilute solutions.

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Anisophylly of Lateral Shoots

BY

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With two Figures in the Text

Abstract

The inequality shown by anisophyllous leaves in plants with decussate phyllotaxis can be measured as the difference in size between the two leaves at a node, expressed as a percentage of their mean size. This is referred to as the *degree of anisophylly*.

In *Acer* anisophylly of two types with different causation occurs.

1. *Primary anisophylly* which is shown only by first year shoots developed from axillary buds and which is due to the inequality of the leaf primordia. Primary anisophylly is thus determined by events occurring during the formation of the bud and is irreversible.
2. *Secondary anisophylly* which is shown chiefly by the second (and subsequent) year's extension growth of lateral shoots from buds which are isophyllous and which is due to the position in which the shoot develops. This type of anisophylly is reversible if the position of the developing shoot be changed.

Effects of secondary anisophylly may be superimposed on those of primary anisophylly.

The recognition of these two causally distinct types of anisophylly occurring in one plant does much to resolve the apparently conflicting information available relating to lateral anisophylly.

Introduction

ANISOPHYLLY of some lateral shoots has long been known. Spencer (1865) was apparently the first to draw attention to the anisophylly shown by the lateral shoots of certain plants with decussate leaves and to the connexion between this condition and external factors especially light. The term anisophylly was first applied by Wiesner (1868) whose definition stated that the leaves lying upon the upper sides of prone shoots have smaller dimensions than those upon the under side, whilst the lateral ones are intermediate. Goebel (1900) recognized that this definition was too restricted and stated that 'by anisophylly we mean that leaves of a different size and of a different quality appear on different sides of plagiotropous shoots. The leaves which stand upon the upper side are usually smaller than those on the under side but the converse is also sometimes the case.'

Goebel reviewed the occurrence of anisophylly in the various plant groups and recognized two types. (1) Plants in which the lateral shoots of radial (isophyllous) chief shoots are anisophyllous. This he designated *lateral anisophylly*. (2) Plants in which the whole shoot system is anisophyllous. This condition was referred to as *habitual anisophylly*. The two conditions are probably not to be sharply separated.

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LATERAL ANISOPHYLLY

Lateral anisophylly, which may cause the shoot to have a distinct dorsiventral appearance, is to be seen most clearly in lateral branches of trees and shrubs with opposite and decussate leaves, such as *Aesculus*, *Sambucus nigra*, *Fraxinus*, and *Acer*. Herbaceous plants with decussate leaves, e.g. *Urtica* and *Scrophularia* may also show it. In plants with spiral phyllotaxis, such as *Quercus*, lateral shoots may also have a dorsiventral appearance, but anisophylly is usually not so obvious as in plants with decussate leaf arrangement.

Anisophylly of lateral shoots may be obscured by twisting of the petioles or by torsions of the internodes. While considerations of anisophylly generally apply to leaves it may be exhibited by prophylls and by bud scales.

From almost the first observations on anisophylly writers on the subject have been concerned with the causes producing this effect. Early work was mainly related to the operation of external factors such as light and gravity. Goebel (loc. cit.) reviewed this earlier work and concluded that of 'earlier views . . . that which made anisophylly a consequence of the influence of gravity is untenable in its generality'. He found that the development of anisophylly in silver fir (*Abies*) although already induced in the bud was strongly influenced by light. Frank (1868) working with *Picea* and *Acer* found that when twigs were twisted through 180° and allowed to grow the original anisophylly was maintained in *Picea* but reversed in *Acer*. Rosenvinge (1889) recognized that in certain instances, e.g. *Taxus*, *Abies*, *Thuja*, and other coniferae, the dorsiventral organization of the lateral branches may be reversed while in other instances it is not reversible and is determined solely by internal causes.

Weisse (1895) studied the anisophylly of Norway maple (*Acer platanoides*) and concluded that the larger size of the under leaf was not due to shading (i.e. a weak etiolation effect) nor to a one-sided nutritional effect due to its position on the shoot. He regarded it as an inherited peculiarity for which a causal explanation could not be offered. It has long been known that in some instances the foliar primordia of axillary buds are unequal in size and thus give rise to anisophyllous shoots, e.g. *Aesculus* (Goebel, loc. cit.).

The genus *Acer* has been much used in studies on anisophylly. Sinnott and Durham (1923) carried out a statistical study of leaf dimensions, using 1,000 leaves from horizontally growing shoots of a maple tree (*Acer saccharum*). They found that all the leaves of a group, i.e. two nodes, had the same blade thickness so that if light intensity determines blade thickness it suggests that on a given twig the leaves in the four positions receive approximately the same degree of illumination, and light is therefore unlikely to be the causal factor bringing about the anisophylly. Sinnott and Durham considered that the most likely factor was gravity since differences in the degree of anisophylly shown by leaves on different shoots appeared to be related to the orientation of the twigs to the horizontal.

In *terminal buds of horizontal shoots* examined during the winter, the authors found that the foliar primordia were equal in size; visible anisophylly was not apparent until the shoot began to grow. It was possible to reverse the anisophylly by twisting a horizontal stem through an angle of 180° before the bud opened.

The present writer (White, 1955) in an investigation of the stem apex of first-year seedlings of sycamore (*Acer pseudoplatanus*) described the formation of axillary buds. The first pair of foliar primordia of the axillary bud is orientated at right-angles to the plane of the leaf axil and the two primordia are equal in size: the second pair of primordia is in the plane of the leaf axil and the two primordia are, in this case, unequal in size. This is true for all the pairs of primordia subsequently formed in the plane of the leaf axil. The smaller primordium is always the one adjacent to the main axis (Fig. 1). Evidence was offered that both the orientation of the primordia and the inequality of those in the plane of the leaf axil resulted from the spatial conditions obtaining in the bud. These axillary buds produced lateral shoots which displayed well marked anisophylly.

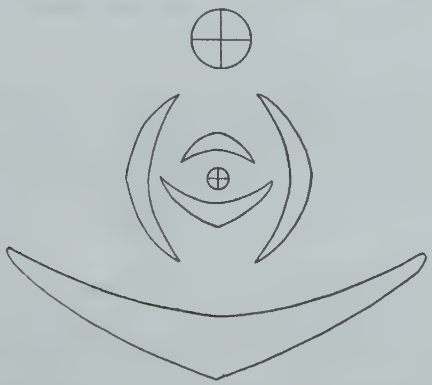

FIG. 1. Plan of an axillary bud of *Acer pseudoplatanus*. The primordium adjacent to the main axis is smaller than the one adjacent to the axillant leaf.

One may perhaps be forgiven for thinking that much of the evidence relating to lateral anisophylly is confusing and the conclusions of different writers conflicting. However, I believe that in the genus *Acer* at least, it is possible to resolve the conflicting views.

MEASUREMENT OF ANISOPHYLLY

Both Goebel (loc. cit.) and Sinnott and Durham (loc. cit.) refer to the degree of anisophylly, or the intensity of anisophylly, meaning apparently the amount of inequality shown by the anisophyllous leaves. It seems desirable to have a means of expressing the amount of inequality rather more precisely. This it is possible to do with plants in which the leaves are arranged decussately. Considering the two leaves at a node, the difference in size between them can be expressed as a percentage of their mean size. This percentage, I propose, should be called the *degree of anisophylly*. The size of the leaves may be measured by their area, by their total length, the length of the lamina, or by the length of their petioles. The length of the lamina is perhaps the most convenient to use since it is quicker to obtain this rather than the lamina area. Petiole length is the least satisfactory since the petiole is the most variable part of a leaf.

OBSERVATIONS ON *ACER PSEUDOPLATANUS*

Main shoots

If the leaves on the main axis of a young sycamore are examined it will be found that only rarely are the two leaves at any node exactly the same size. Normally there is a slight difference in size between the members of a pair, so that on the definition given above there is a certain small degree of anisophylly shown by the main shoot.

In Table I are given the measurements of the lamina and petiole lengths and the degree of anisophylly for the main shoot of a two-year-old sycamore seedling which had been grown in a garden. At the time the measurements were made this shoot had produced 17 nodes, but the leaves at the 17th node were too minute to be measured. The two leaves at each node are referred to as leaf A and B.

The measurements in this table suggest that immature leaves show a greater degree of anisophylly as measured by the lamina length than the leaves which have completed their development. In general the measurements of the petioles are much more variable, and the percentage difference between them is usually greater, than for the corresponding laminae.

Lateral shoots

A different condition obtains in a first-year lateral shoot produced by the extension growth of an axillary bud. In these buds the leaf primordia which are in the plane of the leaf axil are unequal in size. The resultant shoot is markedly anisophyllous. The difference in size between the two leaves set in the plane of the leaf axil is sufficiently great to be obvious on a cursory inspection.

The data given in Table II, obtained from a first-year lateral shoot of a garden-grown plant will serve to illustrate the state of affairs which has been found in all the first-year lateral shoots which have been measured. This plant had 10 nodes at the time the measurements were made.

A comparison of the graphs (Fig. 2) of the degree of anisophylly shows very clearly the difference between main and first-year lateral shoots of *Acer pseudoplatanus*.

The shoot produced by the extension growth of an axillary bud is always anisophyllous, irrespective of the conditions under which the extension growth takes place. This is illustrated in Table III constructed from measurements of an axillary shoot which was growing vertically following the removal of the terminal bud of the plant at the end of the first season. The length of the laminae was measured on three occasions and the degree of anisophylly

TABLE I

Main Shoot of Two-Year Plant

Laminae: length in cm.

Node	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Leaf A	12·4	11·7	12·5	14·4	*	14·5	9·5	12·9	13·9	10·3	11·4	11·4	10·2	10·5	6·7	1·9
Leaf B	12·9	11·5	12·2	14·2	14·6	14·3	9·3	14·2	13·9	10·5	10·6	12·2	10·2	11·9	5·8	1·6
Degree of anisophylly	4·0	1·7	2·4	1·4	—	1·4	2·1	9·6	0	1·9	7·3	6·8	0	12·5	14·4	17·1

Petioles: Length in cm.

Node	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Leaf A	17·8	14·1	15·3	17·1	*	11·8	13·4	15·4	13·4	12·4	10·2	7·9	7·0	3·9	2·1	1·2
Leaf B	20·3	12·4	16·4	15·1	14·5	14·3	13·0	14·4	13·6	12·2	10·5	7·8	7·2	4·6	2·1	1·3
Degree of anisophylly	13·1	12·8	6·9	12·4	—	19·2	3·0	6·7	1·5	1·6	2·9	1·3	2·8	16·5	0	8·0

Nodes 14 onwards were still immature.

* This leaf had fallen.

calculated each time. This axillary bud had four pairs of bud scales; the leaves at the first node were transitionals; they were leaf-like (markedly anisophyllous) and with very wide, expanded petioles.

TABLE II

First-Year Lateral Shoot

Laminae: length in cm.

Node	1	2	3	4	5	6	7	8	9	10
Leaf A	*	7·5	8·1	10·4	10·0	12·2	12·7	13·4	4·4	2·4
Leaf B	7·2	4·3	7·7	6·4	9·1	7·0	10·9	5·6	3·5	1·6
Degree of anisophylly	—	54·2	5·1	47·6	9·4	54·2	15·3	82·1	22·8	40·0

* This leaf had fallen.

It should be noticed that while this shoot shows anisophylly the degree of anisophylly is rather lower than for a lateral shoot growing in its normal position (cf. Table II). This reduction in the degree of anisophylly is probably not without significance.

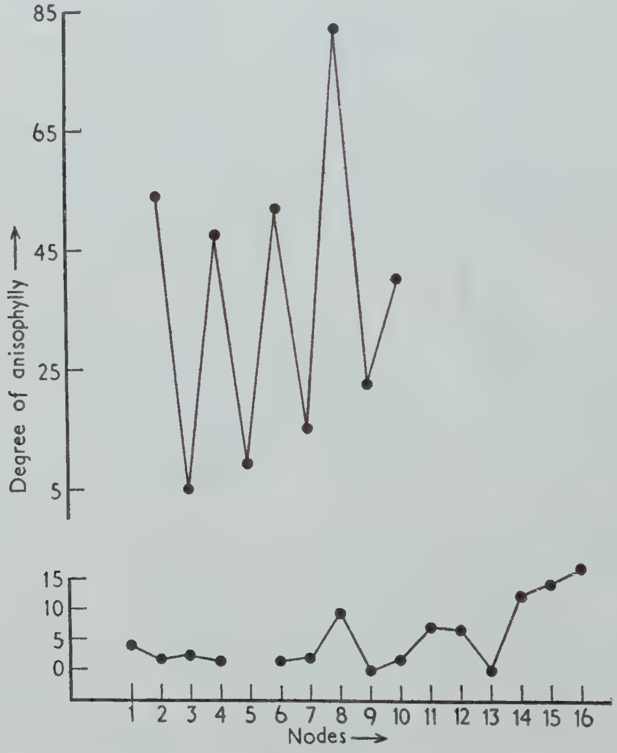

FIG. 2. The degree of anisophylly shown by successive pairs of leaves on a first-year lateral shoot (top) and on a main shoot (bottom), of *Acer pseudoplatanus*.

The lateral shoot developed from the axillary bud eventually forms a terminal (winter-resting) bud. Examination of sections of these terminal buds shows primordia more or less equal in size and strictly comparable to the terminal bud of a main shoot. This observation agrees with that of Sinnott and Durham. While they did not distinguish between lateral shoots of the first and subsequent years when collecting leaves for their investigation, they state (p. 284) that the buds they examined were the terminal buds of horizontal shoots.

In seedling sycamores when the terminal buds at the end of the first-year lateral shoots extend (in the third season) the shoots produced show varying degrees of anisophylly. In some instances it is little more than that shown by the main axis. It should be borne in mind that in young sycamores the lateral shoots often grow at only a slight angle to the main axis and with leaves arranged in four rows. There is no tendency towards the dorsiventral appearance due to petiolar torsions that is seen later in the life of the tree, when the laterals grow in a more horizontal position. When the second-year laterals were growing in a more horizontal position they appeared to show a greater degree of anisophylly. It was presumably with such second (or subsequent) year laterals that Sinnott and Durham obtained reversal of anisophylly, since they wrote of 'twisting a horizontal stem through an angle of 180°'.

TABLE III

Degree of Anisophylly Shown by Erect Growing Lateral Branch

Calculated on lamina length in cm.

Node . . .	1	2	3	4	5	6	7
May 14 . .	157	8·5	41·3	11·1	†	†	†
May 27 . .	157	11·7	24·3	12·5	15·4	0	†
July 15 . .	*	9·3	21·3	9·0	11·2	2·1	36·2

At nodes 1, 3, 5, and 7 the plane of the opposite leaves was that of the leaf axil.

* Leaves shed. † Leaves not yet measurable.

Acer therefore exhibits anisophylly of two types. These we may distinguish as *Primary* and *Secondary* anisophylly. Primary anisophylly is shown by first-year lateral shoots and arises inevitably because the foliar primordia in the bud are unequal in size. The inequality of the primordia and therefore the anisophylly is thought to be due to the spatial conditions within the terminal bud while the axillary bud is forming (White, 1955). Primary anisophylly is fixed and irreversible; it is always the leaf adjacent to the main axis which is the smaller. There is, however, some evidence that the degree of anisophylly finally exhibited by the shoot may be affected by a change in the position under which the shoot develops (cf. Table III). Secondary anisophylly occurs in shoots developing from an essentially isophyllous bud and is induced on the shoot as its extension growth takes place. In extreme cases the shoot will have a truly dorsiventral appearance. Secondary anisophylly is reversible (cf. Sinnott and Durham) and appears to depend upon the orientation of the developing shoot in relation to the earlier axis.

It must be recognized that it is probable that the effects of secondary anisophylly can be superimposed on primary anisophylly. This can perhaps be seen in a negative fashion in a first-year lateral shoot growing vertically, where the degree of anisophylly is lower than in one growing in its normal position (compare Tables II and III).

While no experiments have been carried out on young sycamores to determine the causation of the secondary anisophylly, nothing has been observed that conflicted with Sinnott and Durham's conclusion that it was due to gravity rather than to light. So far as I am aware no one has considered whether the distribution of auxins may play a part in inducing secondary anisophylly. This is a matter which might repay investigation since auxins have been considered to be concerned in the causation of reaction wood, another dorsiventral phenomenon to be seen in lateral branches (Wershing and Bailey, 1942; Sinnott, 1952). The opening buds would provide a source of auxin.

The analysis applied here to *Acer* will probably be found to hold for other plants with a similar phyllotaxis and with unequal primordia in their axillary buds.

VARIATION IN THE DEGREE OF ANISOPHYLLY

Some of the measurements made upon the shoots of sycamores (e.g. Table III) suggested that the degree of anisophylly changed during the development of the leaves. It was thought to be high in the early stages of development and to fall as the development proceeded, but with the recognition of the two types of anisophylly with different causation it is obvious that such an interpretation can only be tentative, since effects due to secondary anisophylly may be imposed on those of primary anisophylly. This matter can only be decided by observations on first-year lateral shoots growing under precisely controlled conditions.

If the anisophylly of a shoot is purely primary then one might anticipate on theoretical grounds some such change in the degree of anisophylly during development. In this connexion is should be noted that commonly the leaves at the base and the apex of a shoot show the highest degree of anisophylly. The basal internodes and leaves of many plants are often anomalous in various ways. This is true of *Acer pseudoplatanus* and Dormer (1950) reported it in *Vicia faba*. The basal leaves of the shoot cease growth at an earlier developmental stage, while the growth of the uppermost leaves on a shoot is often terminated prematurely.

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Studies on Extension Growth in Coleoptile Sections

I. The Influence of Age of Coleoptile upon the Response of Sections to IAA

BY

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With five Figures in the Text

ABSTRACT

One of the problems involved in carrying out large-scale coleoptile cylinder straight growth assays is the effect of coleoptile age upon the behaviour of the sections. This effect, as measured both by the length of coleoptile and the time from sowing the seeds, has been investigated by an examination of the growth of sections cut from coleoptiles of several length grades on five occasions 6–10 hours apart. The response of these sections to a range of concentrations of β-indolylacetic acid (IAA) was followed during the period of growth to ascertain the most suitable time for measurement of sections in a routine test.

A number of general trends associated with coleoptile age (whether measured by length, or time from planting) were observed in amount of growth, growth-rate, period of growth, and sensitivity. It is suggested that these characteristics are associated with the amount of the total growth already made by the parent coleoptile at the time of taking the section, and also that the section exhibits an 'intrinsic growth-rate' related to the growth-rate of the coleoptile at the time the section is cut.

A period of growth of 17–20 hours seems satisfactory for routine tests except where very young coleoptiles are used; in this case growth less than that in water is sometimes observed in very low concentrations of IAA.

INTRODUCTION

IN surveying the growth promoter and inhibitor status of woody shoots by the coleoptile straight growth assay method described in a preliminary paper (Hancock and Barlow, 1953), it was found desirable to compare several extracts in a single test rather than to spread the comparisons over a number of occasions. This meant that 400–600 tubes, each containing five coleoptile sections, were often used in a test, and even with a team of four workers it was impossible to collect the 2–3,000 sections required in less than 4–5 hours; indeed the last sections might be cut as much as 10 hours after the first ones.

Carrying out these large tests thus raised the problem of uniformity of behaviour of sections cut at intervals of some hours from a single sowing of seeds. It is known that sections cut from long coleoptiles are less responsive to growth promoter than those cut from short ones (e.g. Bentley, 1950), but there might still be an effect of coleoptile age on section response even though a restricted grade of coleoptile length is employed throughout. Such a possibility is hinted at by Kefford (1953) and Bennet-Clark and Kefford (1954).

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An extensive examination was therefore made of the growth of sections cut from coleoptiles of several length categories on five occasions 6–10 hours apart, all derived from a single sowing of seeds. Response curves were obtained over a wide range of concentrations of β-indolylacetic acid (IAA). Time-response curves were constructed by measuring the sections at intervals, because experience had suggested that the routine measurement after 17–20 hours in the test solution might not always be the most satisfactory for estimating all concentrations of growth promoter.

MATERIALS AND METHODS

The general procedure was that used as a routine technique in this laboratory.

Wheat seeds (variety Squarehead Master) are germinated in petri-dishes for about 2 days at 6° C., and then grown in trays of coarse sand at 23° C. until the seedlings have reached a convenient size. The coleoptiles are then removed from the seeds, lined up on wet microscope slides, and separated into length grades each of 5 mm. range. (Occasionally seedlings are found in which the leaf does not completely fill the coleoptile, leaving an empty space at the tip; these are rejected, as preliminary work has shown that sections taken from such coleoptiles are less responsive than those from normal seedlings). The graded coleoptiles are again aligned on wet slides, and 10 mm. sections are cut from them by inverting the slide on to a two-bladed cutter which removes the apical 3 mm. and the base. These sections are placed in bundles of five on moist filter-papers in petri-dishes and kept until the required number has been assembled.

All the preceding operations are carried out in phototropically inactive light (Kodak OA yellow filter), and subsequent ones in diffuse daylight, or fluorescent lighting, in the laboratory.

In the operation subsequently referred to as 'entubing' one bundle of five sections is put with 0·5 ml. of test solution into each of a set of small specimen tubes ($2 \times \frac{3}{8}$ in.), which are then closed with waxed corks and rotated horizontally about their long axes on a clinostat about 24 times per hour. After a given period the tubes are removed from the clinostat, and the sections measured with a ruler to the nearest half mm. while still in the tubes.

For this experiment, subsequently referred to by its index number, Test No. 60, a single batch of seedlings was used from which samples of coleoptiles were cut on five occasions, A, B, C, D, and E, separated by intervals of 6, 7, 8½, and 10 hours respectively. On each of these occasions twenty-four bundles of coleoptiles were gathered from as many as possible of the following five length grades, referred to by the Roman numerals I–V: viz. I, 15–20 mm.; II, 23–28 mm.; III, 30–35 mm.; IV, 38–43 mm.; V, 45–50 mm. Grade I was scarce by occasion D, and completely absent by occasion E, at which time grades II and III were also in short supply. Grade IV became available only by occasion C, and V only by occasion E.

The twenty-four bundles of sections from each grade provided for the examination in triplicate of solutions of the sodium salt of β-indolylacetic acid at concentrations of 0, 0·001, 0·01, 0·1, 1, 10, 100, and 200 p.p.m., diluted from a stock solution of 400 p.p.m. with distilled water passed through a resin column (Biodeminrolit).

Measurements were taken for each tube of sections eight times during the course of growth. Full details of the timing of these operations are given in Table I.

TABLE I

Schedule of Operations

Occasion	Cut	Entubed	Measurement times							
	(Hrs. from start)		(Hours from entubing)							
A	0–2	2–2½	½	1¾	3¾	7¾	17¾	25	44¾	69¾
B	6–8½	8½–9	1	2¼	4	8¾	16	26¼	39½	63½
C	13–15½	15½–16	1	2¼	4	8	16	30¾	35	56¼
D	21½–23½	23½–24¼	1½	2¼	4¼	8	12	25	46	69¼
E	31½–34	34–35	1¼	3	—	12	19	36	61¼	109½

RESULTS

The growth response of sections from each grade of coleoptile obtained on each occasion of sampling is shown in the sixteen solid diagrams of Fig. 1. In each diagram the IAA concentrations are shown on the left on a logarithmic scale, increasing from 0 at the front to 200 p.p.m. at the back. Time is shown on the right in periods of 4 hours, with 'zero time' at the front. The actual growth-data (percentage increase over original section length) were plotted as in Fig. 4, for each measurement made, and smooth curves then drawn through the points, and the 4-hourly intervals interpolated.

A certain overall pattern emerges from an appraisal of the results as a whole. In the first place it will be seen in Fig. 1 that the general growth of the sections (i.e. the average heights of the diagrams) decreases both from left to right (occasions A to E) and from bottom to top (grades I to V), the trend being most pronounced along the diagonal A I to E V. This is summed up in the statement that the growth of sections decreases with advancing age of the coleoptiles from which they have been cut, 'age' being expressed either as 'hours from sowing' or 'length'.

This general trend is shown in all concentrations of IAA and in water, and has been given numerical expression in Table II, which shows the maximum length recorded by sections in water and in 10 p.p.m. IAA (about the optimum concentration). Here it has been possible to include values for D I and E II for which solid diagrams for the full concentration range could not be constructed owing to shortage of appropriate coleoptiles.

It will be seen that the greatest percentage increases in length, attained in 10 p.p.m. IAA, are about 110 per cent., achieved by shortest grade coleoptiles on occasions A to C, and by grade II on occasion A, the values falling away both with increasing length of coleoptile and advancing time of sampling. The youngest shortest group, A I, shows the greatest growth in water with a considerable reduction both with longer grades and later occasions, reaching a very low value for E V.

Another general trend in the results shown in Fig. 1 is the change of shape of the time curves; for sections from the youngest sources, these show steep slopes which are maintained for a long time, while with increasing age of source, the slopes are reduced and flatten more abruptly. This trend is given numerical expression for sections in 10 p.p.m. IAA in Tables III and IV. These show respectively the early growth-rates, as given by the slopes of the growth-curves at 1 hour from the start, and the times at which the growth-rates fall to a value of 1 per cent. per hour.

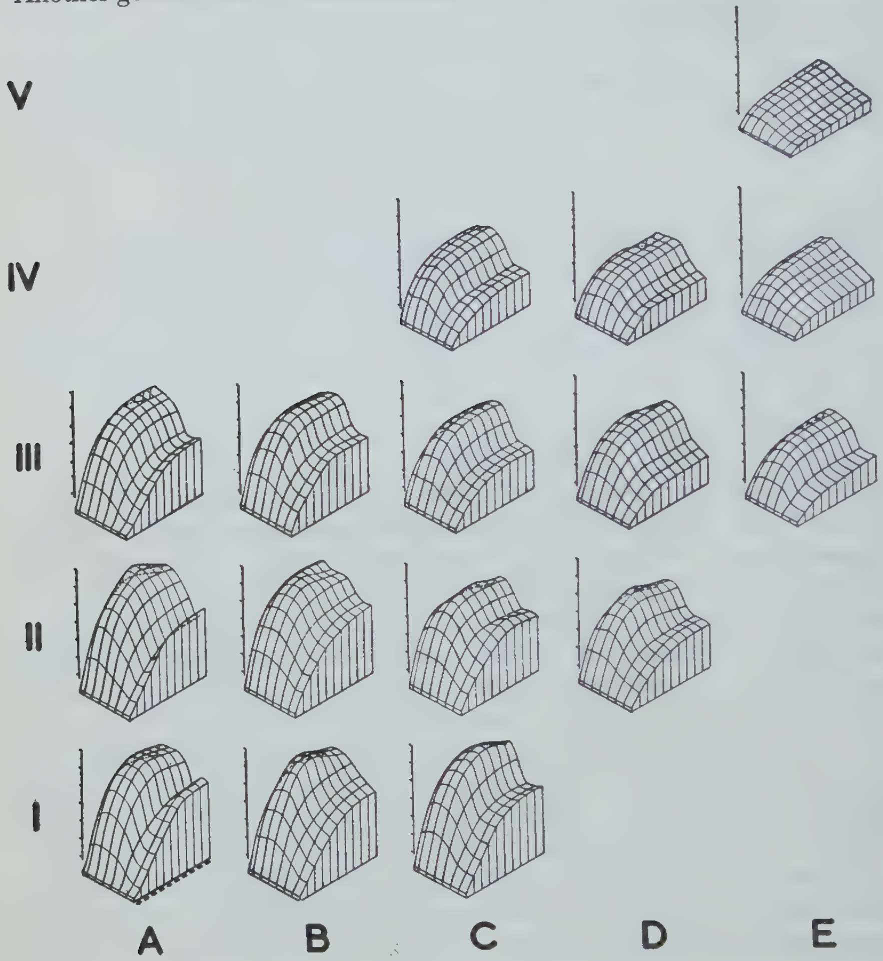

Fig. 1. Smoothed time and concentration response curves for sections of coleoptile cut on five occasions A–E, and of five length grades I–V. On each figure 4-hourly intervals are shown on the right, and concentrations of IAA on a log scale on the left: these are, from front to back, 0, 0·001, 0·01, 0·1, 1, 10, 100, and 200 p.p.m. Percentage increase over original length of the section (10 mm.) is shown on the vertical scale, marked at 20 per cent. intervals.

It will be seen that young coleoptiles have an early growth-rate of between 11·8 and 13·6 per cent. per hour, which does not fall to 1 per cent. per hour for at least 20 hours; the early growth-rate, however, and the time for which it is maintained above the 1 per cent. per hour level both decrease as the coleoptile ages (e.g. with increasing length, I to V, or with the time from sowing, A to E).

TABLE II

Maximum per cent. increase in Length Attained in Water and 10 p.p.m. IAA Solution

Grade of Coleoptile	IAA p.p.m.	Occasion				
		A	B	C	D	E
V	0	—	—	—	—	13
	10	—	—	—	—	24
IV	0	—	—	37	27	18
	10	—	—	64	46	40
III	0	64	61	46	31	28
	10	93	92	78	67	54
II	0	75	77	65	47	42
	10	112	101	93	82	78
I	0	87	71	74	66	—
	10	108	108	112	94	—

TABLE III

Early Growth-rates (per cent. per hour) of Sections in 10 p.p.m. IAA solution

Grade	Occasion				
	A	B	C	D	E
V	—	—	—	—	2·6
IV	—	—	8·8	6·2	4·8
III	10·4	11·4	8·6	8·2	7·0
II	11·8	12·6	10·8	10·8	8·8
I	13·4	13·6	11·4	8·8	—

TABLE IV

Time in Hours at which Growth-rate of Sections falls to 1 per cent. per hour in 10 p.p.m. IAA Solution

Grade	Occasion				
	A	B	C	D	E
V	—	—	—	—	7
IV	—	—	15	11	11
III	21	19	18	16	15
II	24	20	18	16	18
I	21	21	23	25	—

The numerical expression given to the trends shown in the above two tables has been made possible by the use of an equation relating length of coleoptile section, y, with the time in the test solution, x: $y = a+b(1-e^{-kx})$. This relation fits the observed points excellently for the 10 p.p.m. concentration of IAA. It should be mentioned here that extrapolation of the time-growth curves of Fig. 1 to zero time is not legitimate for these data, because storage of the sections after cutting had resulted in variable amounts of growth taking place before the sections were added to the test solution. It is for this reason that the diagrams of Fig. 1 (or as shown more clearly in Fig. 4) start with a low vertical face at the left, and not from zero. However, in a number of cases (particularly for the young and short type of coleoptile) although an excellent fit is obtained for all the observed points for sections in 10 p.p.m. IAA by using the above equation, the intercept on the y axis falls below the original starting length of the sections. Furthermore it has been found that although some of the response curves for section growth in water can be fitted by this equation, there are a number in which a good fit to the data occurs only over a limited part of the curve, the observed growth-rates in the earlier stages being much lower than the calculated ones. Although it is probable that in the case of sections in IAA the low early growth-rates may be due to a lag period while the IAA is entering the section (Housley *et al*, 1954), in water such an explanation cannot hold, and it is thought that the observed rates may be connected with characteristics of the coleoptile from which the section was cut. This matter will be dealt with later in this paper.

Complementary to Fig. 1, which shows the increase in length of the various types of sections during the first 40 hours in the test solution, Fig. 2 presents the rates of growth over the same period. The actual data are here plotted —not the smoothed curve values—so that the duration of the individual periods over which rates of growth can be calculated vary somewhat. To simplify the diagram, only rates in water and in 10 p.p.m. IAA are shown, and, for reasons to be mentioned later, the first period shown is that between the time of the first measurement and the one made about four hours from the start.

The same general pattern is evident as has been shown by the calculated values in Tables III and IV; high rates are maintained for the longest time by sections from the youngest, shortest coleoptiles, while low rates are briefly held by sections from the oldest, longest sources. Upon more detailed examination, however, certain other features emerge. For example, it is noticeable that in most cases after 8 hours the rates in water and in IAA are very similar, the higher early rates thus largely accounting for the differences in final lengths. Again it will be seen that the youngest sources yield sections which show an early high growth-rate in IAA maintained with little change for 8 hours (A), while older categories show a much reduced rate after 4 hours (B I–III and C I) or, with still further advance in age, never achieve high rates at all.

In contrast to the behaviour in IAA, in water, sections from the younger sources exhibit steady or even rising growth-rates during the first 16–20 hours (A I–III, B I, C I and during 12 hours in D I), while in the older groups there is a steady decline in growth-rates (B II and III, C III and IV) or, if there are indications of a rise, then the values are always low (D II–IV).

Over such a wide concentration range of growth promoter—a 200,000-fold increase from 0·001 to 200 p.p.m.—the linear relation usually claimed for this assay method, between elongation and the logarithm of the concentration, does not hold; for all times of measurement in all types of section the relation is in fact curvilinear, with a small response to low concentrations of IAA, a larger one over the more usually investigated range between about 0·01 and 1 p.p.m., and a flat region above this followed by a decreasing response at 100 or 200 p.p.m. Response curves obtained over a wider range, both in this and other laboratories, bear out the relation shown here.

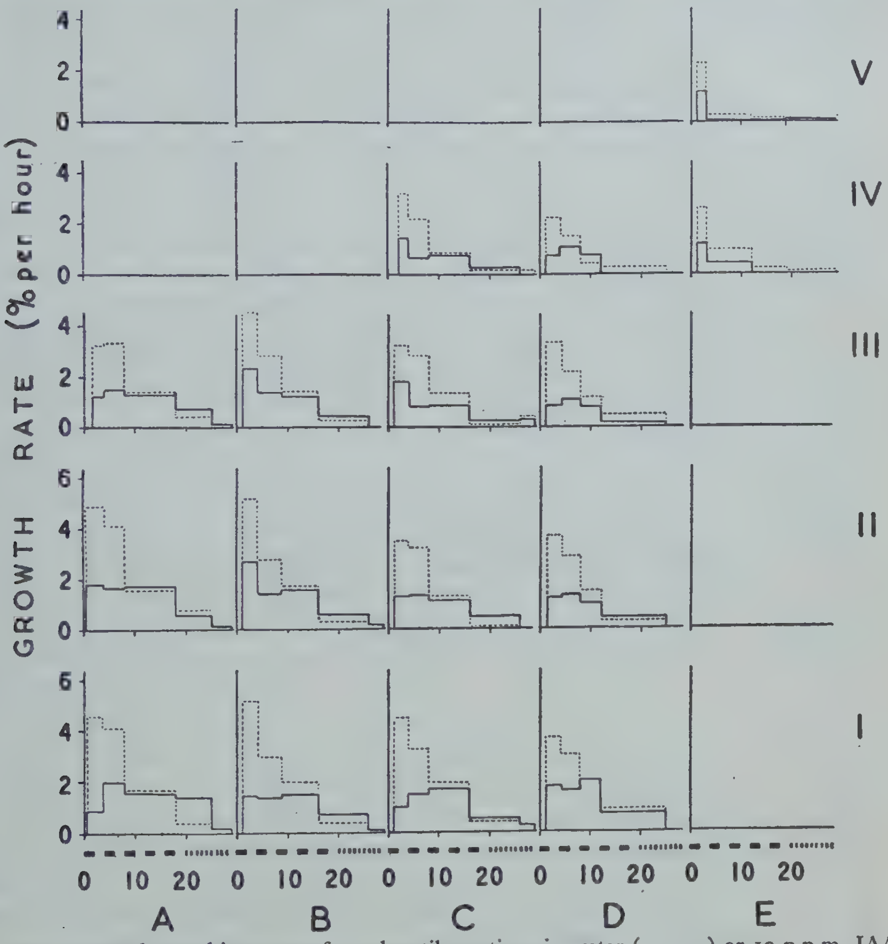

FIG. 2. Growth-rate histograms for coleoptile sections in water (———) or 10 p.p.m. IAA (·········) gathered on occasions A–E, and of length grades I–V. The time-scale has been contracted after 20 hrs. To facilitate rapid comparison between the various time-scales on the diagrams in this paper, 2-hourly intervals are indicated throughout by alternate black and white lines.

In sections from the youngest shorter coleoptiles, A I and II, later measurements actually show less growth in low concentrations of IAA than in water, resulting in the development of a trough in these solid diagrams.

These features are illustrated in Fig. 3 which shows the response curves of sections from the series across the diagonal of Fig. 1 from A I to E V,

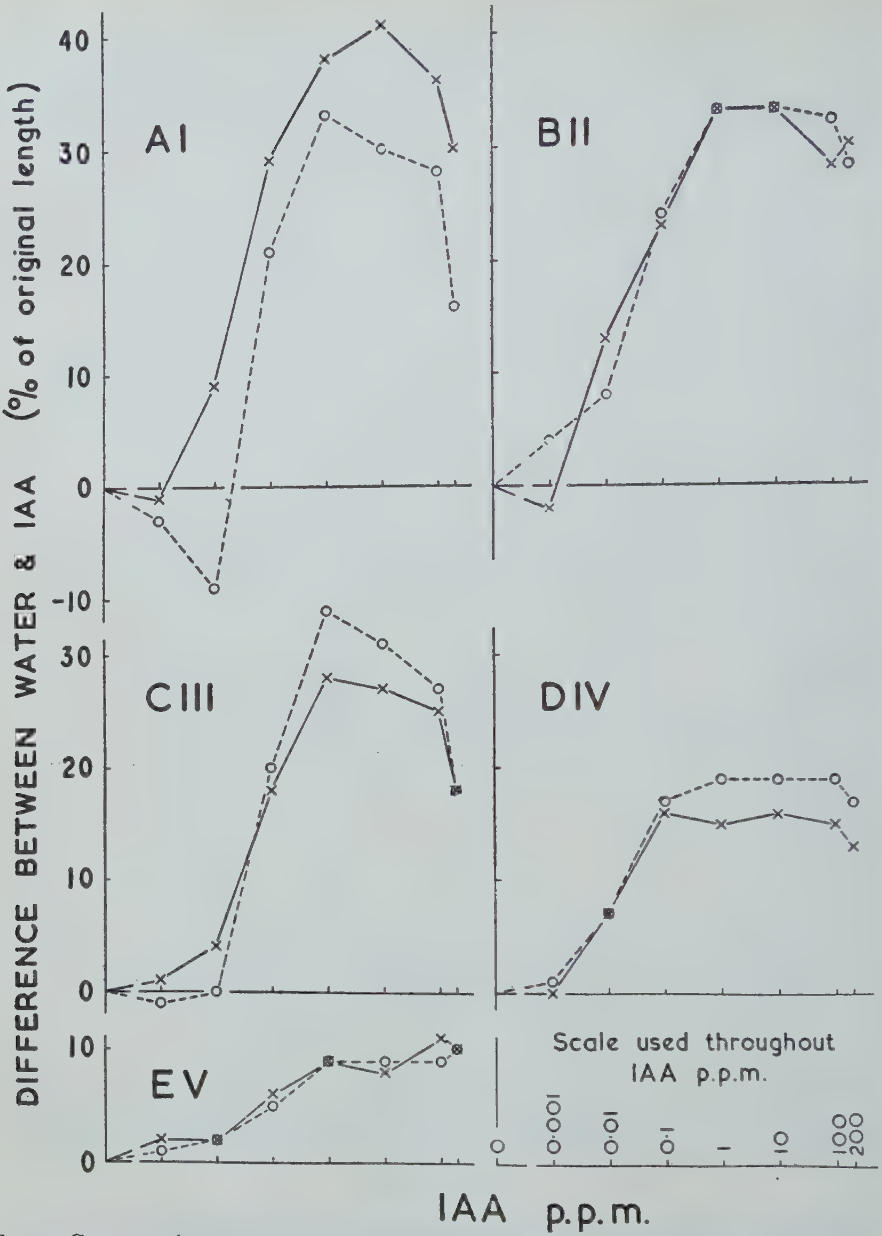

FIG. 3. Concentration response curves of sections of types A I, B II, C III, D IV, and E V (i.e. the diagonal of Fig. 1), as differences in percentage increase in length in water and IAA, after 8 hours (x———x) and 20 hours (o··········o) in the test solution.

exemplifying the trends in the complete data. The pairs of curves are drawn from the measurements made at or near 8 and 25 hours from the start. Because in an assay method of this kind growth in the test solution is usually compared with that in water, in Fig. 3 the response curves are presented as differences between growth in water and each IAA solution, the units being percentage of the original section length.

These curves exemplify four trends with age of the coleoptiles providing the sections. (1) There is a decrease in 'sensitivity' to IAA over the range of concentrations from 0·01 to 10 p.p.m. on passing from the young to the old coleoptiles (e.g. A I to E V) shown both in height and the slope of the curves. (2) At low concentrations of IAA, for most section categories the measurements made at 8 to 25 hours give much the same picture, but this is not so for the young short type, A I. In this case the earlier measurement shows a distinct positive response to 0·01 p.p.m. IAA, but the later one shows an equal negative response—i.e. the sections have grown less than in water during the intervening period. The way in which this change-over takes place is illustrated in Fig. 4; the significance of this effect will be discussed later. (3) At near-optimum concentrations of IAA, while in the youngest types the 8-hour measurement differs more from water than does the 25-hour one,[1] as the age of the coleoptile increases the values first coincide,[2] and then reverse—i.e. the difference at 25 hours is greater than at 8 hours.[3] Finally in E V the values converge again, all being low. (4) At supra-optimal concentrations of IAA the response is most depressed in the younger types and least in the older (or not at all in E V).

In general, then, it will be seen that the most suitable type of section for use in this assay method to some extent depends upon the kind of information required. For example, if extreme sensitivity is needed over a narrow range of concentrations, then young short coleoptiles provide sections which should be suitable for this purpose if measured early; (should practical difficulties preclude an early measurement, then such coleoptiles should be avoided). If, on the other hand, a wide concentration range must be covered, then older longer coleoptiles would be more suitable (provided the potential response is large enough for easy measurement), and time of measurement is not critical.

It is of interest to note in Fig. 3 that even in the highest IAA concentration of 200 p.p.m., growth never falls below that in water, in contrast to the behaviour of oat coleoptile sections (mentioned for example by Bennet-Clark and Kefford (1954) and Housley, Bentley, and Bickle (1954)).

It should be mentioned here that in none of the sections in this test had any shrinkage taken place by the time of the latest measurement shown in Fig. 3, though shrinkage did occur in most cases later on. This feature of behaviour also followed a general pattern associated with the type of coleoptile providing the sections. It was found that sections from the youngest types of coleoptile shrank earlier than those from older types; e.g. shrinkage was observed in 10 p.p.m. IAA within 50 hours from the start for section types A and B I to III, C and D I and II, but nearly double that time elapsed before sections from types E IV and V were affected. In the highest concentration

[1] e.g. A I in Fig. 3, and A II and III, not shown.

[2] e.g. B II in Fig. 3, and B I and III, not shown.

[3] e.g. C III and D IV in Fig. 3 and C I, D II and III, and E IV not shown.

used in this experiment (200 p.p.m.) the times at which shrinkage took place were similar to those at the 'optimum' (for growth) of 10 p.p.m. In water the sections showed no signs of decreasing in length for much longer times, shrinkage being observed in the younger types between 50 and 70 hours from

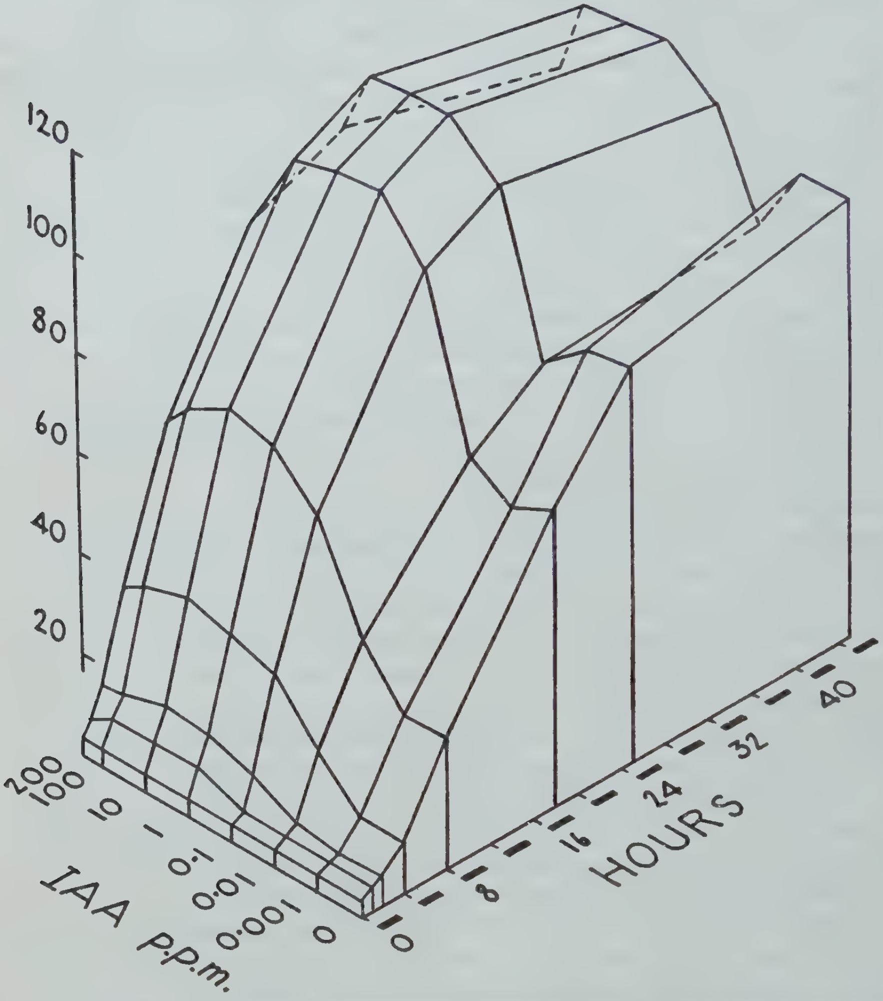

FIG. 4. Time and concentration response curves for sections from the youngest and shortest coleoptiles, A I, to show the development of the 'subnormal' response to low concentrations of IAA seen as the trough at 0·01 p.p.m. from 24 hours onwards. The vertical scale shows the percentage increase over original length. (From this and similar diagrams for the other section types, the smoothed curves of Fig. 1 were drawn.)

the start while in the older types E II–V and D IV, it was not observed until much later, the oldest type, E V continuing in a healthy state for over 110 hours.

Although sections eventually become waterlogged and obviously flaccid, shrinkage often occurs before such gross visible changes have taken place, so that the 'normal' appearance of a section is no certain criterion of a healthy condition, a point to which attention has already been drawn by Housley *et al* (1954).

DISCUSSION

This experiment was undertaken to answer two of the questions which had arisen during large-scale assays: (1) What is the influence of age of coleoptile upon section response? (2) Is the growth-interval of 17–20 hours, adopted as routine, a satisfactory one for all types of section?

As regards the first point, the results present a good picture of the ways in which coleoptile age affects the response of sections, and have shown that 'age' must be reckoned both in terms of time from sowing, and of length of coleoptile, a long coleoptile cut early behaving in a similar way to a short one cut, say, 15 hours later.

In general the results may be summarized by contrasting the behaviour of sections cut from young coleoptiles with that of sections cut from old ones, in the following respects: increase in length is greater, both in water and in all concentrations of IAA tested (Table II); supra-optimal concentrations of IAA are more depressing (Fig. 3); growth-rates in IAA are higher (Table III), and in both IAA and water are maintained longer (Table IV and Fig. 2); shrinkage of sections occurs earlier.

It is considered that these results may all be related to two particular characteristics of the coleoptiles at the time of gathering—their growth-rates, and the proportion of their final length attained at that time.

To obtain information on these points the growth of a batch of seedlings was observed under similar conditions to those obtaining in Test No. 60. At first, samples of seeds were removed and examined, and then, as soon as coleoptiles appeared above the surface of the sand, 175 were labelled (by dropping numbered rings over them) and measured at intervals. The individual records were sorted into length grades for each occasion of measurement, and were found to fall naturally into several groups, for which averages were obtained. Five of these curves are shown in Fig. 5, the others taking up their appropriate places in between. A second batch of seedlings yielded another series of measurements which gave very similar results.

Fig. 5 shows only that part of the curves falling between 70 and 150 hours from sowing, and on them a grid of lines has been superimposed to show the mid-points of the periods of gathering equivalent to occasions A–E of Test No. 60 (vertical lines), and the mid-points of the length grades I–V of that test (horizontal lines). Broken lines running across the growth-curves join those points on each curve at which 50, 70, and 90 per cent. of the total growth has been attained.

It is suggested that it is this heterogeneity of the population as regards growth-rate which accounts for the differences in performance of sections cut from the various types of coleoptile; those coleoptiles which provided the earliest sections not only grew most rapidly but attained the greatest length, while those yielding the latest sections were both slower growing and shorter at the end—i.e. they were not merely late starters which eventually followed a course of growth similar to their predecessors. Again, these curves show how with increasing age of coleoptile the 'growth potential' of the section cut from it declines, whether age be regarded as time (A to E), or length (I to V).

Most important, however, is the variation in the rate of growth of the intact coleoptile, this being higher for 'young' than for 'old' on either scale; on the basis of Test No. 60 it is suggested that these differences are reflected in the growth-rates of the sections taken from these coleoptiles—i.e. sections have an *intrinsic growth rate* related to the growth-rate of their source. This is most likely to appear in sections placed in water, as added IAA accelerates extension thereby masking the expression of the intrinsic rhythm of growth; those coleoptiles sampled before they reach their maximum growth-rate (i.e. below the point of inflexion of their growth-curve) should yield sections which themselves show an increasing rate for a few hours, while sections cut after this point should show a diminishing growth-rate from the start.

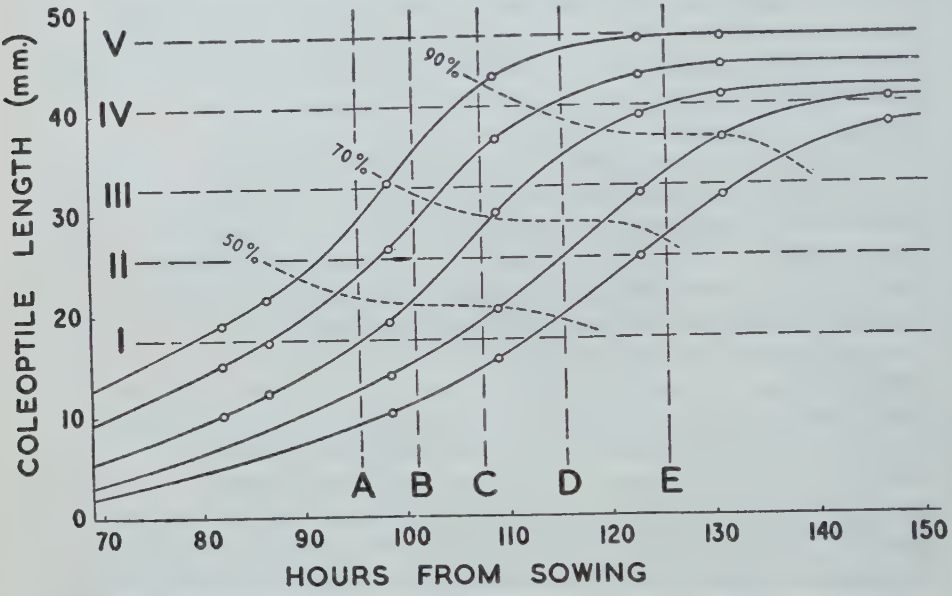

FIG. 5. Part of the growth-curves for wheat coleoptiles on seeds grown in sand, with the positions indicated (..........) at which 50 per cent., 70 per cent., and 90 per cent. of the total growth has been made. The horizontal and vertical lines correspond to the mid-points of the length grades I–V and the occasions of gathering coleoptiles A–E for Test No. 60.

The evidence of Test No. 60 in conjunction with the observations of seedling growth, supports this hypothesis; for example, in Fig. 5 it will be seen that A I–III, B I, and C I are all at or below the inflexion point of the growth-curves, and in Fig. 2 their growth-rates in water are shown either more or less flat or rising; in contrast B III, and C III and IV are above the inflexion point and exhibit falling growth-rates. The agreement is not perfect, B II, for example, showing a sharper fall in growth-rate than would have been expected, and C II the reverse.

A striking and quite independent example of rising growth-rates of sections in water is to be found for *oat* sections in Fig. 1*a* and *b*, of the paper by Bennet-Clark and Kefford (1954), where the rate increases slowly for 15–18 hours. If our hypothesis applies to oat seedlings this would imply that the sections were cut from very young coleoptiles, although it is just possible that some other rhythm has been superimposed on the normal growth-cycle, such as that described by Ball and Dyke (1955) for oat seedlings transferred from red light to darkness (a treatment, incidentally, which they found to be without effect on wheat).

As it seemed possible that the intrinsic growth-rate of a section after isolation might sometimes be quite transient, particularly in the presence of added IAA, it was thought that it should best be indicated by early measurements of the sections. In the experiment described above, however, the sections had been stored for a period before being put into the tubes, and in the few cases where they were measured within half an hour of entubing it was clear that growth of the sections had taken place during the storage period; in other cases, where the first measurements were not made until later, as has already been mentioned, extrapolation of the growth curves did not take them through 'zero percentage increase' at 'zero time' (e.g. Fig. 4) because time was reckoned from the moment of entubing the sections, not of cutting them from the coleoptiles.

The earliest growth measurements were not in fact used in Fig. 2 for the further reason that—particularly in water—the rates of growth in many cases showed a preliminary high value followed by a very low one, after which the rate changed steadily. It was thought that these fluctuations were in some way associated with storage and that the earliest growth-rates did not necessarily reflect the intrinsic rates at all reliably.

Regarding the second question which Test No. 60 was designed to answer—as to the best time of measurement of sections—it seems that this is immaterial in all but sections from the youngest sources; there the usual routine measurement after 17–20 hours may show a negative response to low concentrations of IAA, i.e. growth is actually below that made in water, while an earlier measurement, say after 8 hours, may show a positive response to these same concentrations.

Apart from the practical effect of this observation on the conduct of straight growth assays, the fact that in certain circumstances coleoptile sections extend less in low concentrations of a growth promoter than they do in water has wider implications. It is therefore desirable to consider how often such circumstances arise; in the first place the considerable body of data obtained from the 'control series' included in the survey tests already alluded to was studied and revealed the 'subnormal' response to low concentrations of IAA in 47 out of 197 possible cases (24 per cent.). (Those series were considered as 'possible' if they included concentrations of 0·01 or 0·001 p.p.m. IAA, and otherwise gave satisfactory response curves). In a further 117 cases (59 per cent.) the response curves were ‘typically sigmoid’ without a ‘subnormal’ region, while the remainder (17 per cent.) showed aberrations which did not readily fall into any clear-cut groups. As about a quarter of these routine tests have given indications of subnormal responses to fairly low concentrations of IAA, it was possible that this type of response may occur more frequently at lower concentrations; the effect of very much lower concentrations was therefore specially investigated. The results of one such test showed little effect of these treatments after 5 hours in the solution, but later the establishment of the subnormal response:

p.p.m. IAA		10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1
% increase over water	5 hrs.	−0·5	−2	0	1	8	20	24
	20 hrs.	−3·5	−4	−4	−1	2	11·5	18

In the second place, although the literature has not been systematically searched for evidence of this type of response, instances have occasionally been noticed, e.g. Bentley and Housley (1952) Fig. 6 for 2,4–D; (1953) Fig. 5, which also illustrates the same time sequence noted above in the development of the subnormal response; (1954) Table 5, Expr. D.24.

The only comment noticed is by McRae, Foster, and Bonner (1953) in connexion with their Fig. 5 and Table V, where low concentrations of 2,4–D reduce the growth of sections in low concentrations of IAA below the level attained in IAA alone. They suggest that this kind of antagonism may also occur between applied and native growth promoters, and cite as an example (from Bonner and Bandurski, 1952) the induction of flowering in the pineapple by low concentrations of 2,4–D or NAA; these synthetic growth substances are said to antagonize the endogenous auxin, reducing its effective level below that which inhibits flower formation. This speculation may be extended to the present case, the only necessary assumption being that the native auxin of the wheat coleoptile is at least not entirely IAA. This is not an unreasonable assumption in view of the various growth promoters in addition to IAA which have now been found in several kinds of plant material. The difficulty in this interpretation which has not yet been resolved lies in the effect of the low concentrations not being immediately apparent, but taking many hours to become established.

However, this idea of antagonism between low concentrations of auxins goes far to resolve the paradox that while it is the generally held view that root growth is normally being held in check by supra-optimal concentrations of endogenous auxin, it can nevertheless be *stimulated* by adding further promoter, albeit at very low concentrations. Again the implication is that for an effect of this kind to be observed the applied and the native auxins must be different, but as has just been mentioned, this is quite probable as there is likely to be a mixture of auxins present in most tissues; how these native auxins themselves interact is at present difficult to guess.

It will be seen that the results of this experiment have raised three points of considerable importance to the treatment of auxin-induced growth of coleoptile sections by the methods of enzyme kinetics as described by Foster, McRae, and Bonner (1952). (1) These methods require the determination of 'initial growth-rates' of sections in auxin solutions which is made difficult under our conditions by the curvilinear relationship of growth with time (see also Bennet-Clark and Kefford (1954) and Housley, Bentley and Bickle (1954)). (2) An additional complication occurs when sections from young coleoptiles are measured after some time in the auxin solution, when at low concentrations growth less than that in water is observed. (3) By whatever method initial rates are determined there remains, at least at low concentrations of exogenous auxin, the problem of the intrinsic growth rate of the section, i.e. the growth-rate in the absence of added promoter, which upsets the relation between initial growth-rate and auxin concentration. Further discussion of these aspects will appear in the second paper of this series.

ACKNOWLEDGEMENTS

Test No. 60 involved the presence of at least three persons during a continuous period of 72 hours, and the authors wish to acknowledge the willing help given by Mr. J. Hanford, while acting as a Temporary Recorder at East Malling Research Station, who by augmenting permanent staff, made it possible to adhere to the complicated time-table of operations. The authors are also indebted to Miss J. G. Smith for the considerable amount of extra work involved, and for the help of the Statistics Section in fitting the exponential relationship to the time curves discussed in this paper.

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Studies in the Physiology of Parasitism

XXIV. Further Experiments on the Killing of Plant Cells by Fungal and Bacterial Extracts

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With five Figures in the Text

ABSTRACT

When cucumber and turnip tissues were subjected to enzyme preparations of *Botrytis cinerea* and *Bacterium aroideae* in the presence of plasmolysing concentrations of various crystalloids, killing of cells was retarded out of all proportion to the slight retarding effect on maceration. When the tissue cells were recovered from plasmolysis they regained their sensitiveness to the toxic action. Toxicity of mercuric chloride and oxalic acid was also much reduced when cells were plasmolysed.

Fractional precipitation with various proportions of acetone and over a wide range of pH gave preparations which varied in activity from one treatment to another, but their macerating and toxic actions varied together.

After partial maceration, plant tissues were much more sensitive to the toxic action of mercuric chloride or oxalic acid. This is interpreted as meaning that, before maceration is complete, the toxic principle has been able to reach the protoplastic surface and to exert a deleterious effect upon it.

The evidence presented gives further support to the view that the enzyme system of the two pathogenic organisms which macerates the host cell walls also brings about death of the protoplasts.

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INTRODUCTION

THE present paper is a continuation of investigations carried out by Tribe (1955) who examined, among other things, the effect of certain chemical substances on the rates at which cell-walls were disintegrated and protoplasts were killed by preparations derived from two pathogenic organisms, *Bacterium*

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aroideae and *Botrytis cinerea*. Tribe found that concentrations of salts or non-electrolytes, which were sufficiently strong to plasmolyse the cells of the tissue being tested, greatly reduced the killing action of the preparations used, this effect being out of all proportion to their retarding action on rate of cell-wall disintegration (maceration) or of degradation of pectin solutions. This was the first record of a distinct quantitative difference being established between enzymic action on the cell-wall and toxic action on the protoplast. Such a result could be interpreted as indicating that the macerating and killing agents are different. There is, however, the alternative hypothesis that the active principle is the same for both maceration and killing, but that the process of plasmolysis affects the outer protoplasmic membrane in such a way as to protect its sensitive constituent from the action of the macerating enzyme. The fact that strong retardation of the killing effect was equally brought about by isosmotic concentrations of various salts, and of non-electrolytes such as sucrose, glucose and urea, suggests that the action of these is more likely to be on the substrate itself than directly on the toxic substance.

MATERIALS AND GENERAL METHODS

These were substantially the same as described by Tribe. The organisms used were *Bacterium aroideae* and *Botrytis cinerea*, as in the earlier paper. Active enzymic preparations were obtained by incubation of the organisms at 25° C. in flasks containing a shallow layer of the nutrient medium (glucose, 20 gm.; asparagin 5 gm.; mineral salts as in Tribe's formula; water, 1 litre). With cultures of *Bacterium aroideae*, 1 per cent. calcium carbonate was added to the medium to prevent the development of a retarding degree of acidity in the culture during early stages of growth. After 5 days' incubation the medium of the *Botrytis* cultures was filtered off, bulked, divided into 20 ml. portions in stoppered bottles and stored at −18° C. until required. Five-day-old cultures of *Bacterium aroideae* were centrifuged at 8,000 r.p.m. for 5 minutes in order to remove the calcium carbonate and the bulk of the bacterial cells, after which the treatment was the same as for the *Botrytis* preparation.

Enzymic or macerating activity was determined with tissue discs (potato, turnip, cucumber fruit) of standard thickness (0·3 or 0·5 mm.) cut on a microtome. For each test, 10 discs were used and the average time in minutes (R.T.) for loss of coherence to take place was determined. Activity was expressed as A_m, derived from the formula

$$A_m = \frac{1}{\text{R.T.}} \times 1{,}000.$$

Progress of the toxic action was followed by the plasmolytic method of Tribe (1955, pp. 353–4). At appropriate intervals, 3 discs were transferred from the preparation under test, placed in a watch glass, and 1 ml. of the plasmolysing mixture (molar KNO_3, 8·5 ml.; 0·1 per cent. neutral red chloride, 1·0 ml.; veronal buffer at pH 7·4, 0·5 ml.) added. Readings were taken after the discs had been in the plasmolysing solution for 20–30 minutes.

The method of recording results was based on a numerical rating from 0–5 according to the estimated proportion of cells killed, i.e. of cells which were no longer plasmolysable and the protoplasts of which did not take up the red dye. The ratings were:

0—all cells dead; no red spots visible to naked eye or to hand-lens magnification.

1—occasional cells alive, scattered red spots.

2 → 3 → 4—increase in number of red spots, and finally

5—discs uniformly red, this being the reading obtained with healthy untreated discs.

Such a rating was called the 'neutral red index' by Tribe. From a time series of such indices one could calculate the time required for half the cells to be killed. This may be called the D50 point, corresponding to a neutral red index of 2·5. The toxic activity of the preparation was then expressed by the formula

$$A_t = \frac{1}{\mathrm{D50}} \times 1{,}000.$$

For pH control, the preparations were buffered with veronal buffer according to Michaelis' data as given by Gortner and Gortner (1949). Reactions were carried out at the respective optima, viz. at pH 5·5 for *Botrytis cinerea* and 8·5 for *Bacterium aroideae*.

PLASMOLYSIS AND DEPLASMOLYSIS OF CELLS

As has already been stated, Tribe showed that living cells, when plasmolysed, became much more resistant to the toxic action of preparations of *Bacterium aroideae* and *Botrytis cinerea*. In the present work the analysis has been carried one step farther and the effect of deplasmolysis has also been studied. This was done in order to determine whether the increased resistance exhibited by tissues in plasmolysing solutions is a temporary one due to the physical effects of plasmolysis, or whether some permanent change is brought about by such treatment and the cells cannot again be restored to their natural behaviour.

Considerable difficulty was encountered in the earlier experiments because of the large percentage of cells which were killed in the deplasmolysing process. This was later found to be caused by too rapid a change in the concentration of the solution during the plasmolysing and deplasmolysing treatments. When these manipulations were carried out more gradually, successful recovery from plasmolysis was finally achieved.

A particular example of plasmolysis and successful deplasmolysis, viz. with cucumber discs in solutions of potassium nitrate, will illustrate the successive stages of the process:

(*a*) Discs immersed in 0·1 M. KNO_3 for ½ hour (incipient plasmolysis); then in 0·3 M. KNO_3 for ½ hour (strong plasmolysis);

(*b*) Solution diluted to 0·1 M. and allowed ½ hour; then by successive steps in 0·075, 0·05, 0·01 M., one ½ hour in each, and finally washed in tap water. The discs regained full turgidity and responded to the neutral red plasmolysis test as well as did untreated discs.

Table I gives the results of two experiments with cucumber discs and *Bacterium aroideae* preparations. The Table is in two parts, corresponding to the plasmolysing agent used. The method of experiment was the same for both, so that the precise method adopted for potassium nitrate only need be described.

TABLE I

Effect of Plasmolysis and Recovery from Plasmolysis on Macerating and Toxic Actions of Bacterium aroideae *Preparation. Test Discs, Cucumber*

Agent and concentration	R.T. (min.)	Neutral Red Index at intervals										Estimated D50 point
		Minutes						Hours				
		10	20	30	45	60	90	2	4	6	24	
A. Potassium nitrate												
0·0	23	—	2	2	1	0	—	—	—	—	—	20 min.
0·05	26	—	5	4	2	1	0	—	—	—	—	40 min.
0·075*	26	—	—	5	—	5	5	5	3	1	0	4 hr.
0·1	29	—	—	5	—	5	5	5	5	3	0	6+ hr.
0·2	30	—	—	5	—	5	5	5	5	5	3	24+ hr.
A′. (Recovery)												
0·1	29	—	—	5	—	5	5	5	5	3	0	6+ hr.
0·075	29	—	—	5	—	5	5	5	3	1	0	4 hr.
0·05	30	—	5	4	2	1	0	—	—	—	—	40 min.
0·01	30	—	3	3	1	0	—	—	—	—	—	30 min.
0·0	29	—	3	2	1	0	—	—	—	—	—	25 min.
B. Sucrose												
0·0	17	2	1	0	—	—	—	—	—	—	—	10 min.
0·1	17	2	1	0	—	—	—	—	—	—	—	10 min.
0·2	17	3	1	1	0	—	—	—	—	—	—	15 min.
0·3*	17	—	5	5	—	5	—	5	3	2	—	5 hr.
0·4	17	—	5	5	—	5	—	5	4	2	—	6 hr.
0·5	17	—	5	5	—	5	—	5	4	2	—	6 hr.
B′. (Recovery)												
0·4	17	—	5	5	—	5	—	5	5	3	—	6+ hr.
0·3	17	—	5	5	—	5	—	5	5	3	—	6+ hr.
0·2	17	3	2	1	1	0	—	—	—	—	—	20 min.
0·1	14	2	1	0	—	—	—	—	—	—	—	10 min.
0·0	13	2	1	0	—	—	—	—	—	—	—	10 min.

* Concentration at which plasmolysis was first evident.

Solutions of concentrations over the range 0–0·4 M. were prepared, with and without enzyme. With solutions containing the enzyme, the same concentration of enzyme was present in all. About 40 discs were placed in each of the solutions without the enzyme and left for 20–30 minutes. These discs were then used for testing the activities of the enzyme solutions at the corresponding salt concentrations. For determination of enzymic activity, 10 discs were placed in 1 ml.; for following the rate of killing, 20–30 discs were placed in 2–3 ml., depending on the number of tests to be made, and 3 discs were used for each estimation.

Concomitantly with these tests, a large number of cucumber discs were brought, in two steps, into 0·2 M. KNO_3, maintained there for ½ hour, then brought back gradually to the condition shown under the first column of A′ in Table I. They were then transferred to enzymic solutions containing the same salt concentration.

Essentially, therefore, part A of Table I gives the behaviour of fresh discs which have been subjected to a given enzyme concentration at varied salt concentrations; part A′ gives the parallel behaviour of discs which had first been plasmolysed in 0·2 M. KNO_3 and then brought back to the appropriate salt concentration. The same applies to parts B and B′ for sucrose as plasmolysing agent.

The work represented by the two parts, AA′ and BB′, of Table I was done on different days with different enzyme preparations and different lots of cucumber discs; hence some variation—viz., 23 and 17 min. respectively in A and B for the reaction time of enzyme on fresh discs in the absence of any salt.

The concentration at which plasmolysis of the cells of cucumber tissue first became visible was 0·075 and 0·3 M. for KNO_3 and sucrose respectively. This was determined by careful microscopic examination of discs taken from each of the various treatments prior to treatment with the enzyme solutions.

Table I shows that discs which have been strongly plasmolysed behave like fresh discs when they are partially or fully recovered from plasmolysis. The data under A′ and B′ in the Table are substantially the mirror image of the data under A and B respectively. It is also clear that with increasing concentration of the plasmolysing agent the time necessary to achieve killing of the protoplasts increases more rapidly than the reaction time for maceration. This result is illustrated more fully in Fig. 1 which includes the relevant data from Table I together with the results of another experiment in which the plasmolysing agent was magnesium sulphate.

Similar results were given with turnip discs as test material, the main difference being the higher concentration of crystalloid required for plasmolysis (0·6 M. with sucrose).

Experiments with *Botrytis* preparations gave the same type of result, but less pronouncedly, as is seen from Table II.

TABLE II

Effect of Osmotic Concentration on Macerating and Toxic Actions of Botrytis cinerea *Preparation. Test Discs, Cucumber*

Concentration of Potassium nitrate	R.T. (min.)	D50 point (min.)
0·0	23	25
0·05	28	35
0·075	38	75
0·1	40	100
0·2	40	140

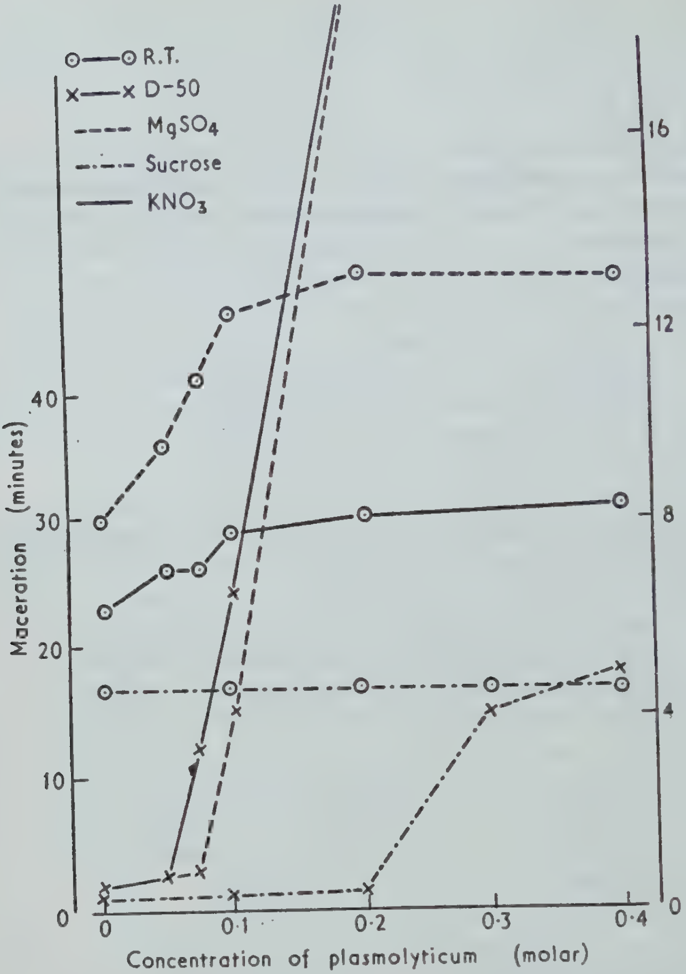

FIG. 1. Effect of plasmolysis on killing action of *Bacterium aroideae* enzyme. Test discs, cucumber.

In 0·075 per cent. KNO_3, which causes incipient plasmolysis, the D50 stage was reached in 75 minutes, which is 3 times that in the water control. The corresponding ratio for *Bacterium aroideae* preparation (Table I, A) was 12. The average ratio in 6 experiments with *Botrytis cinerea*, in which various plasmolysing agents were used, was 3·5; in 8 experiments of a similar kind with *Bacterium aroideae*, it was 16·2.

With salt concentrations which cause strong plasmolysis, e.g. 0·2 M. KNO_3 in Table I, the toxic action of *Bacterium aroideae* preparation is very strongly retarded (at least 70 times in Table I). With magnesium sulphate as plasmolysing agent, the effect is still more pronounced, no obvious killing of protoplasts having taken place even after 24 hours. The corresponding retardation with *Botrytis* preparations is much less, the D50 point rarely exceeding about 6 hours.

The difference in question is at least partly due to the reactions of the *Botrytis* preparations being carried out at pH 5·5, the optimum for its macerating enzyme system, as against pH 8·5 for those of *Bacterium aroideae*. An acidity represented by pH 5·5 is harmful to cucumber tissue cells, as shown by the fact that there is appreciable killing after 24 hours in the controls containing only the acidified plasmolysing agent. Similar results are reported by Tribe.

The retarding effect of plasmolysis on the toxic action of *Bacterium aroideae* and *Botrytis* preparations is paralleled by a similar effect on the toxic action of oxalic acid and mercuric chloride, as is shown by the following results. Discs of cucumber tissue were placed in 0·1 per cent. solutions of oxalic acid and mercuric chloride, either as such or in the presence of 0·5 M. potassium nitrate. The D50 points were:

0·1% oxalic acid in water	20 min.
0·1% oxalic acid in 0·5 M. KNO_3	300 min.
0·1% $HgCl_2$ in water	15 min.
0·1% $HgCl_2$ in 0·5 M. KNO_3	240 min.

Whatever the nature of the toxic principle of the fungus or of the bacterium, it is certainly neither of the chemical substances just referred to; and the fact that the action of all three upon living cells is strongly retarded in the presence of a plasmolysing agent definitely favours the view that the retardation of killing is brought about more by an effect upon the protoplasmic substrate than upon the toxic substance itself.

ATTEMPTS TO SEPARATE MACERATING FROM KILLING ACTIVITY

Brown (1915) attempted to separate a macerating from a killing substance by a variety of methods—by heat inactivation, by pH adjustment, by diffusion and dialysis, but without obtaining evidence of any separation; by whatever means the macerating action was affected, so was the killing action and, as far as could be determined by inspection, to much the same degree. That earlier work is amplified here by a number of methods and with the aid of a quantitative means of estimating both killing and macerating actions.

i. *Differential precipitation with acetone*

The acetone was precooled to −10° C. and the crude fungal or bacterial preparation to about 5° C. The mixture was then kept at 5° C. for 1–2 hours to allow the precipitate to collect before it was separated by centrifuging. Precipitations were carried out at pH 8·5 and 5·5 for the preparations of *Bacterium aroideae* and *Botrytis* respectively. Various proportions of acetone to crude preparation were used, the precipitates dried, weighed and taken up in half the original volume of water. The purified preparations were then assessed for macerating and toxic activities as shown in Figs. 2 and 3.

As was to be expected, the weight of precipitate obtained increased with the concentration of acetone in the mixture; in the *Botrytis* preparation the precipitate was much more sparse than in the bacterial one. For both preparations, the curve for macerating activity showed the same general trend as that for toxicity.[1] There was no evidence of a difference in precipitability

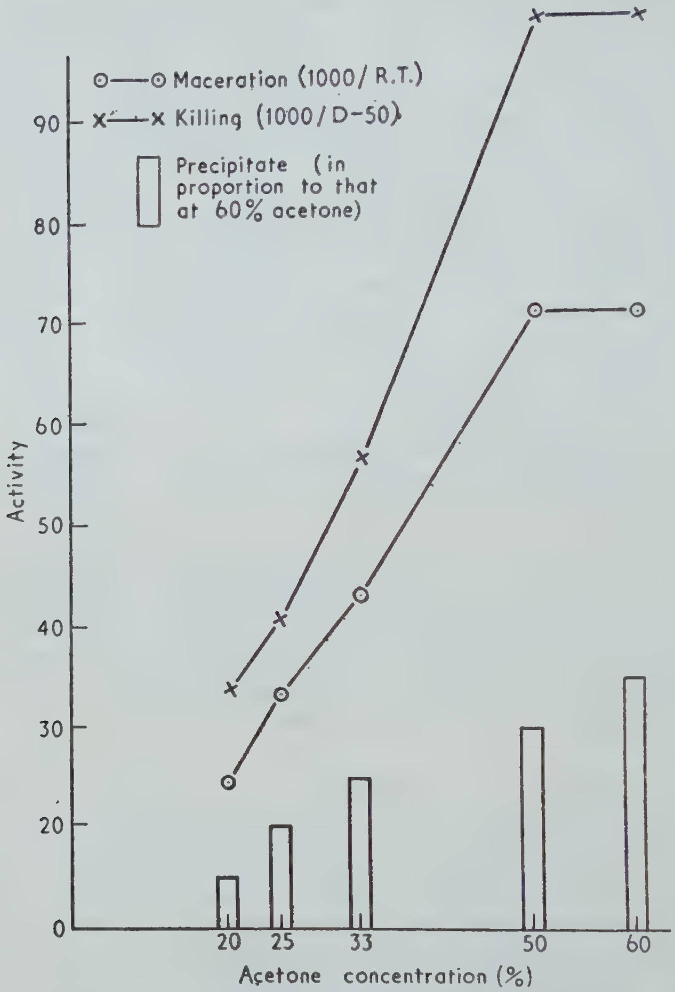

FIG. 2. Effect of acetone concentration on precipitation of *Bacterium aroideae* enzyme. Test discs, cucumber.

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[1] In Fig. 2 the curve for toxic activity is above that for maceration; in Fig. 3 the position is reversed. This is mainly due to a well-established difference between the bacterial and *Botrytis* preparations, viz. that the former has brought about considerable killing of cells at the stage where tissue coherence is lost, whereas the latter brings about loss of coherence at a time when the cells are still alive. The difference is somewhat accentuated by the choice of cucumber discs for the test with *Bacterium aroideae* and of turnip discs for the test with *Botrytis*.

of two substances, one responsible for maceration and the other for toxic action.

The efficiency of precipitation with acetone is also affected by pH adjustment. Table III gives the results obtained when one volume of acetone was added to two volumes of crude *Bacterium aroideae* preparation at various pH values over the range 5–9. The dried precipitates were finally taken up in half the original volume.

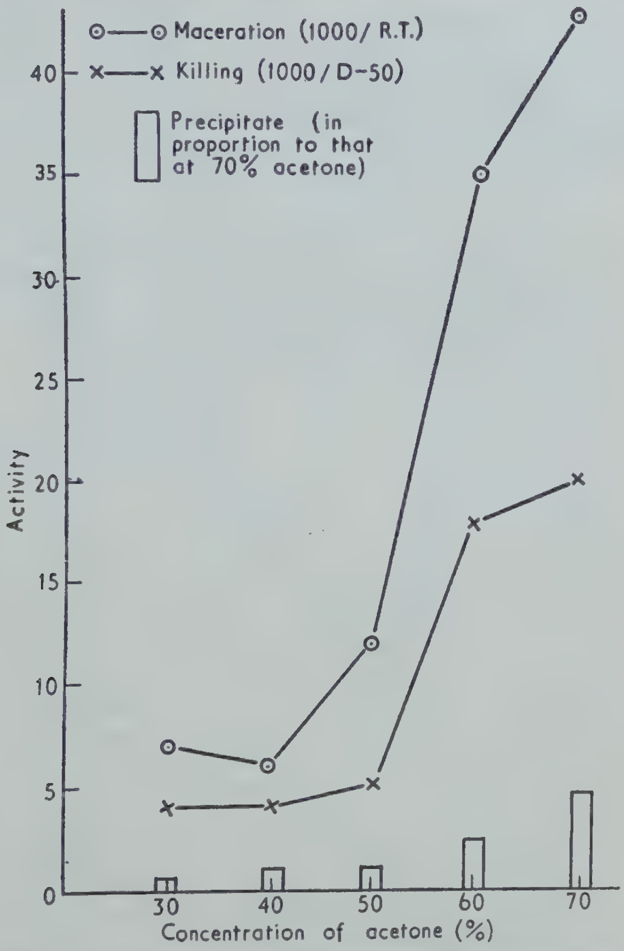

FIG. 3. Effect of acetone concentration on precipitation of *Botrytis cinerea* enzyme. Test discs, turnip.

Here again there was no evidence of a separation of a macerating and a toxic substance, the set of values for R.T. running closely parallel to that for D50.

Precipitation of crude *Botrytis* preparation with acetone (equal volumes of each) at pH's over the range 3–9 gave results in full agreement with those for *Bacterium aroideae.*

TABLE III

Effect of pH on Acetone Precipitation of Bacterium aroideae *Preparation. Test Discs, Turnip*

pH	Nature of precipitate	Wt. of ppte. (mg.)	R.T. (min.)	D50 (min.)
5	Colloid-like, appearing in 2 hrs.	Trace	270	250
6	As above	Trace	195	210
7	Fine particles, appearing in 30 min.	11	25	30
8	Flocculent, settling in 30 min.	16	15	20
9	Flocculent, settling in 15 min.	22	15	20

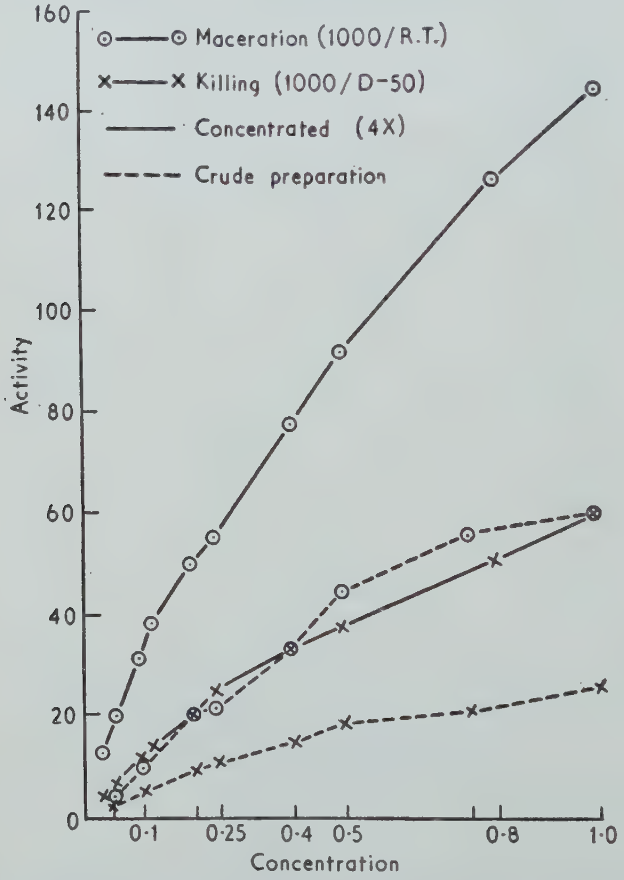

FIG. 4. Effect of dilution on crude and concentrated *Botrytis cinerea* enzyme solutions. Test discs, turnip.

A two-fold precipitation of crude *Botrytis* preparation was carried out with acetone; the purified and concentrated preparation had a macerating activity about twice and a toxic activity about 2·5 times that of the crude preparation. No substantial relative change in these activities was indicated.

ii. *Effect of dilution*

Though dilution, in itself, would not separate a macerating from a killing substance, it might reveal the presence of such if their activities varied with concentration according to different laws. On the chance of this happening a comprehensive series of tests was made with crude and twice-purified (by acetone precipitation) preparations of the two organisms. Fig. 4 gives the results for *Botrytis*; those for *Bacterium aroideae* were essentially similar.

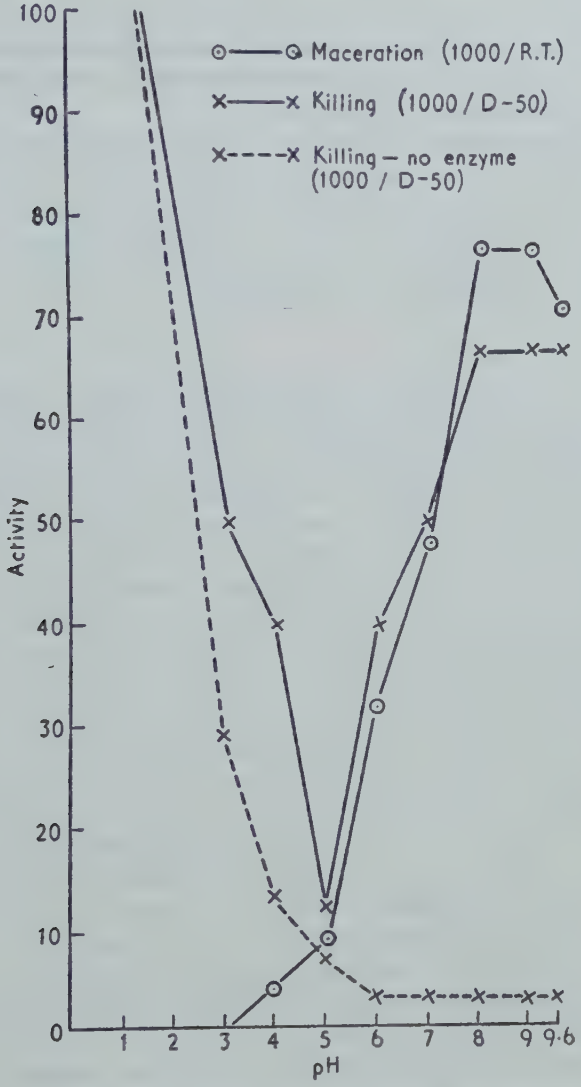

Fig. 5. Effect of pH on *Bacterium aroideae* enzyme. Test discs, cucumber.

It is clear that the curve for toxic activity has the same general trend as that for maceration, and this is true both for crude and for purified preparations. If the scale of the curves for killing is slightly more than doubled, each curve for killing lies close to the corresponding one for maceration. All four curves tend to be concave to the x-axis.

iii. *Effect of pH.*

The rates of toxic action and of maceration were determined over the range pH 3–9 for crude preparations of the two organisms. The curves for *Bacterium aroideae* are given in Fig. 5.

The curves for toxicity and maceration run a parallel course from pH 5 upwards; below pH 5 there is a sharp divergence but, as the control (dotted) curve shows, there is an increasingly pronounced toxic action due directly to acidity. Within the range where the control solutions give slight effect there is good agreement between the two curves. Similar behaviour was shown by the curves for *Botrytis* preparations.

iv. *Effect of partial maceration*

The main technical difficulty in separating a toxic from a macerating agent in the preparations used lies in the fact that the protoplast is within the cell-wall. It is certain that the toxic agent is colloidal, and therefore it may be unable to reach the protoplast until the wall has been decomposed to a certain extent. If this were so, any treatment which retarded or inhibited the macerating action would have a parallel effect on the toxic action, even though the toxin itself might be unaffected by the particular treatments.

In the early experiments on this subject, Brown (1915) attempted to meet this difficulty by allowing maceration to proceed to a fairly advanced stage; he then stopped it by adjustment of pH and observed the effect on the progress of cell killing. He worked with a preparation of *Botrytis cinerea* and with turnip discs as test objects. The sequence of events with these materials is that, at the time when coherence of the discs is lost, all the cells are still alive, but they begin to die soon afterwards. The action was allowed to continue to the stage where coherence was lost and then further action on the cell-wall was stopped by shifting the reaction of the solution to the alkaline side of neutrality. At this stage killing of the cells was about to take place, and therefore one must presume that the toxin was now having access to the protoplasts. It would not be expected, therefore, that the change from a slightly acid to a slightly alkaline reaction would affect the toxin to the same extent as it affected the enzyme, i.e. if the two substances were different colloids. Nevertheless the effect of stopping the enzyme action was to retard very strongly the toxic action. That the latter was not completely stopped is not surprising if, as is likely, the cells had already been damaged to some extent by contact with the toxin.

Further evidence on this point was obtained in this work by experiments from a different angle. The effect of partial maceration of tissue on the rate of cell killing by mercuric chloride and oxalic acid was investigated. For these experiments, *Botrytis* enzyme was used with turnip discs, a combination which gives the most pronounced lag between maceration (R.T.) and killing (D50). For convenience in the manipulations, a relatively weak enzymic preparation (R.T. 60 min.) was used. Discs which had been allowed to macerate 0, 30, and 60 minutes were placed in 0·01 per cent. mercuric chloride or 0·01 per cent. oxalic acid and the time required to reach the D50 stage determined. The data for mercuric chloride are given in Table IV.

TABLE IV

Effect of Partial Maceration with Botrytis cinerea *Enzyme on Toxic Activity of Mercuric Chloride*

Treatment	D50 total	D50 *less* enzyme treatment
A. Enzyme continuous (R.T. 60 min.)	180 min.	120 min.
B. Enzyme for 60 min., then 0·01% $HgCl_2$	75 min.	15 min.
C. Enzyme for 30 min., then 0·01% $HgCl_2$	90 min.	60 min.
D. No enzyme, 0·01% $HgCl_2$	3 hr.	3 hr.

A similar set of results was obtained for 0·01 per cent. oxalic acid.

Both mercuric chloride and oxalic acid are readily diffusible substances which are able to pass through parenchymatous walls, whether these are in the normal or in the partially macerated condition. The rapid killing of the protoplasts when the cell-walls have been brought to the stage where coherence of the tissue is lost, as compared with the much slower killing of intact protoplasts, can be explained only on the assumption that the former have already been seriously affected by some agent. This must be the *Botrytis* toxin. Furthermore, a similar, though not so large, effect is shown with tissue which has been treated with *Botrytis* enzyme to the half-way stage of complete maceration. Therefore, at this stage also one must assume that the toxin has had access to the protoplasts. These results are against the hypothesis that the *Botrytis* toxin is able to act only when maceration is well-nigh completed. From this it follows that the many-sided evidence of parallelism between maceration and toxic effects indicates a very remarkable similarity of properties if enzyme and toxin are different substances.

EFFECT OF ENZYMIC DEGRADATION PRODUCTS

There is a possibility that degradation products of the cell-wall may play a part in killing the protoplasts. The main hydrolytic product of pectic substances is galacturonic acid and its polymers. That toxicity might be due to an acid reaction produced in this manner is ruled out at once by the fact that the optimum for maceration and toxicity by preparations of *Bacterium aroideae* is at pH 8–9. The following experiment was carried out to determine whether any enhanced toxicity was shown by enzyme preparations in which a considerable amount of tissue had been macerated.

About 160 turnip discs (0·8 cm. × 0·5 mm. thick) were immersed in 10 ml. of enzyme preparation of each organism, buffered at pH 5·5 for *Botrytis* and at 8·5 for *Bacterium aroideae*. After complete maceration, the liquids were cleared by centrifuging and a second equal batch of discs added. The process was repeated a third time and was allowed to run overnight. Altogether about 12 cubic centimetres of turnip tissue were digested in an original 10 ml. of enzyme. In the course of these digestions the pH of the enzymes, though buffered, had moved to 5·8 and 6·4 respectively. Neither of these, it may be noted, represents a degree of H-ion concentration which would be rapidly toxic to turnip or cucumber cells.

Both enzymes, and especially that of *Botrytis*, had been considerably weakened in the process of digesting so much turnip tissue. The bacterial enzyme gave an R.T. of 115 min. and a D50 point at about 5 hours; with *Botrytis* enzyme, neither the R.T. nor the D50 stage had been reached in 24 hours. Clearly there was no evidence that a toxic substance had accumulated in the enzyme preparations as a result of the digestion in them of a relatively large mass of plant tissue.

ACKNOWLEDGEMENTS

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Dry-matter Production by *Pinus sylvestris* L.

BY

J. D. OVINGTON

(*The Nature Conservancy, Merlewood, Grange-over-Sands*)

With nine Figures in the Text

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ABSTRACT

This paper contains the data for the gross weight and relative proportions of canopy, bole, and roots in a series of twelve even-aged forest stands.

From these it is possible to describe the growth curves of trees of *Pinus sylvestris* in Britain under the observed conditions and the production of dry matter per unit area of such stands of trees. The following general results are given: regressions relating weight to bole, height, and diameter; the relative growth of leaves, roots, and bole; annual increments of dry matter; relative growth-rates per unit leaf area or per unit dry weight per annum.

Some comparative data are given for *Pinus nigra* (one plantation) which is more productive than *Pinus sylvestris*.

[Annals of Botany, N.S. Vol. XXI, No. 82, 1957.]

INTRODUCTION

AT present most of the timber used for building and industry in Great Britain is imported, and it appears unlikely that future requirements of timber can be met entirely from British woodlands. In view of a rising world demand for wood and the reduction in the forest resources of the world, it is imperative that the maximum amount of timber be produced from the land devoted to forestry. Approximately one-twentieth of the land surface of Great Britain is forest-covered, and plans have been made to more than double the area of productive woodland within the next half-century.

For practical reasons, sylviculturalists measure woodland productivity as cubic volume of stemwood so that relatively little is known of the weight of dry matter produced by trees and particularly of the weights of the associated tree canopies and roots, which are normally of no great economic value. The biological evaluation of dry-matter production should be based essentially upon dry-weight measurements and upon a knowledge of the relative proportions of canopy, bole, and root. The determination of woodland productivity in this way presents a problem more complex than the corresponding estimation for most agricultural crops, since trees by virtue of their perennial and woody habit are able to attain a great age and size. Furthermore, the timber crop is harvested a long time after planting, the harvesting period extending over many years.

Data are presented in this account of the gross weights and relative proportions of canopy, bole, and root for single trees and for the trees as a group in an age series of twelve, even-aged forest stands at the state forest of Thetford Chase. The forest stands are all situated within a compact, flat area of continuous woodland near Brandon, on typical sandy Breck soils as described by Watt (1936). There is probably very little variation in climatic conditions over the whole sampling area. Each stand is composed of a single tree species, eleven of *Pinus sylvestris*, and one of *Pinus nigra*, and all have been planted except two of Scots pine, which have resulted from natural regeneration within cleared compartments.

WOODLAND FORM (Table I, Fig. 1)

A sample plot, usually a quarter of an acre, was marked out in each of the selected woodlands, and within each plot 100 random trees were measured for bole and canopy characteristics. Age is reckoned from the time of germination, the trees being grown for the first 3 years in a nursery before being planted out.

Successive stages of development can be recognized for the plantations of Scots pine. Initially the trees are so small and widely spaced relative to their size that there is little competition with one another, but eventually the canopies meet and the lower shaded branches ultimately die. The presence or absence of green leaves was used as the criteria as to whether a branch was alive or not. The height zones of living and dead canopy differentiated in this

TABLE I

Average Tree Dimensions in Sample Plots

Tree spp.	Grown as	Age yrs.	Ht. cm.	No. trees per hectare	Ht. lowest living branch cm.	Ht. lowest dead branch cm.	Stem diameter at breast ht. cm.	Minimum canopy spread cm.	Maximum canopy spread cm.	Ht. maximum canopy spread cm.
Pinus sylvestris	Seedling plants	3	33	5,310	—	—	—	—	—	—
Pinus sylvestris	Plantation	7	136	4,810	7	5	0·5	23	60	47
,, ,,	,,	11	285	4,230	29	13	3·5	49	133	91
,, ,,	,,	14	359	5,190	21	4	4·2	56	149	107
,, ,,	,,	17	485	5,640	84	11	5·7	60	211	121
,, ,,	,,	20	576	5,400	151	18	6·8	67	304	136
,, ,,	,,	23	823	3,640	377	171	9·4	74	438	162
,, ,,	,,	31	1,262	2,370	689	277	13·9	81	819	215
,, ,,	,,	35	1,419	1,890	835	264	14·8	66	931	209
,, ,,	,,	55	1,601	760	1,068	573	22·7	80	1,205	264
,, ,,	Natural regeneration	11	225	58,010	39	4	1·4	17	110	49
,, ,,	,, ,,	14	432	27,750	186	5	3·1	35	292	78
Pinus nigra	Plantation	31	1,319	1,680	537	377	17·9	101	871	199

way are not sharply separated since individual whorls may contain both live and dead branches. As the trees become taller the zone of living branches is raised higher above ground level and there is a corresponding upward extension of the lower dead canopy. By the twentieth year many of the lowermost branches have been lost from the bole, either artificially or naturally, to form a branch-free space below. Associated with these changes in tree form there are corresponding changes of stocking density; in the young plantations the stocking density is relatively constant varying with factors such as the success of planting and the number of invading natural seedlings, but when cropping begins after the twentieth year, a marked and continuous decrease in the number of trees per hectare occurs.

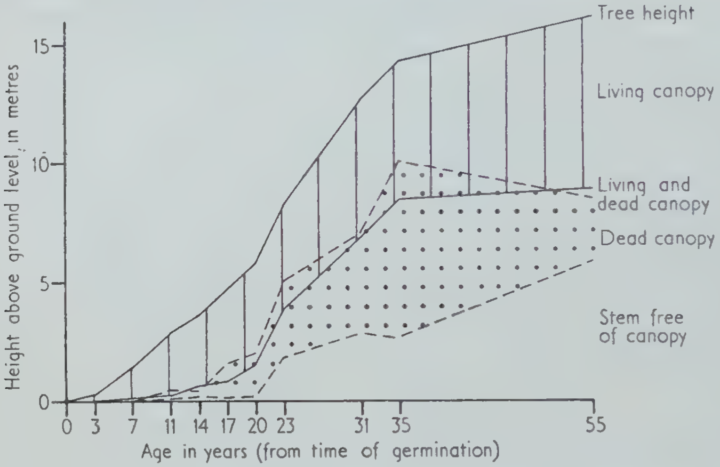

FIG. 1. The development of *Pinus sylvestris* grown in plantations

Yield tables for Scots pine have been prepared by Hummel and Christie (1953), and in general the plantations correspond to their Quality Class II for stocking density and Quality Class I for height. The fact that the stocking densities are higher than is usual for Quality Class I may only be a reflection of the selection of plantations, since only plantations in close canopy that had not been recently thinned were investigated. Height growth of the plantations gives a sigmoid curve, the average height increasing rapidly in the close canopy plantations but not so rapidly at 50 years of age. The height of the oldest plantation is less than would be expected of Quality Class I, for height increment does not decline in this class until after 70 years of age. The poor height growth of the oldest plantation probably reflects its different history of management since the woodland was not planted by the Forestry Commission but by private owners on the open Breck for shelter and sport. Bole diameter growth unlike that of height is maintained throughout the 55-year period.

The natural regeneration is extremely abundant, with about ten times the number of seedlings present than would be planted normally. In these highly competitive conditions mortality is heavy and not more than 70 per cent. of the trees survive to 14 years of age. Lateral canopy spread is severely restricted and the trees have shorter lengths of living canopy and thinner stems than trees grown in plantations.

When the plantations of *Pinus sylvestris* and *Pinus nigra* of similar age are compared, the average tree in the Corsican pine plantation is larger in both canopy and bole dimensions, and due to the heavier cropping the stocking density is less than that of the Scots pine.

METHODS

Although the trees in each plantation are of identical age and have grown from similar stock under comparable environmental conditions, some natural variations occur between individual trees. In fact the forester classifies trees according to their size and form and usually selectively thins the different classes so as to favour the more desirable trees. Ideally each group should be sampled separately, but since the number of trees that could be handled was limited and the variability within a stand was not unduly large, sample trees corresponding as closely as possible to the average tree dimensions for the plots were selected for detailed mensuration.

The sample trees were felled in mid-August 1954 and carefully wrapped in hessian for protection before being transported by lorry to the laboratories. In all, 21 sample trees were taken, 3 in each of the plots of natural regeneration, 2 in all plantations up to 20 years old, and 1 in each of the remaining plantations. Twelve seedling plants of a similar age to those that were originally planted were also measured and weighed. Each sample tree was separated into leaves, branches, and bole, and the dry-matter content was determined after drying representative samples to a constant weight at a temperature of 80° C.

The collection of the roots of the sample trees was delayed until the following December, when the main tap-root and all roots over 0·5 cm. diameter were carefully extracted from the soil by hand digging and winching. Rootlets with a diameter of less than 0·5 cm. were sampled on an area basis by taking a number of soil cores through the rooting depth of each plot and separating the small roots from the mineral soil. The dry-matter contents of the root systems were determined as for the shoots.

All weights given in this account are oven dry.

SAMPLE TREES OF *PINUS SYLVESTRIS* IN PLANTATIONS

1. *Canopy*

(*a*) Cones (Table II, Fig. 2)

Cone formation is known to be very variable (Holmes and Matthews, 1951), depending upon climatic and other factors, so that in some years cone production is high whilst in other years hardly any cones are formed. The sampling year is probably fairly typical in the relative amounts of cone carried by the average trees in plantations of different ages. Cones are occasionally present

TABLE II

Cones and Branches on Sample Trees

	Age yrs.	Total wt. of cones kg.	Total wt. of living branches kg.	No. of living branches	Av. wt. of living branches g.	Total wt. of dead branches kg.	Total wt. of branches kg.	Total wt. of needle-bearing shoots			No. needle-bearing shoots			Av. wt. needle bearing shoots		
								1st yr. kg.	2nd yr. kg.	3rd yr. kg.	1st yr. (× 10)	2nd yr. (× 10)	3rd yr. (× 10)	1st yr. g.	2nd yr. g.	3rd yr. g.
Plantations of *Pinus sylvestris*	7	0	0·20	28	7	0	0·20	0·09	0·05	0·04	21	11	4	4·3	4·5	10·0
	11	0	1·02	47	22	0·01	1·03	0·23	0·29	0·24	51	45	24	4·5	6·4	10·0
	14	0·01	1·50	43	35	0·27	1·77	0·28	0·30	0·23	65	64	47	4·3	4·7	4·9
	17	0·13	1·67	38	44	0·45	2·12	0·22	0·31	0·31	78	61	38	2·8	5·1	8·2
	20	0·32	2·22	43	52	1·21	3·43	0·25	0·34	0·45	118	81	34	2·1	4·2	13·2
	23	0·11	3·80	56	68	3·58	7·38	0·15	0·40	0·44	216	138	82	0·7	2·9	5·4
	31	0·09	3·97	66	60	5·67	9·64	0·36	0·41	0·52	176	113	75	2·0	3·6	6·9
	35	0·38	5·08	51	100	8·07	13·15	0·36	0·47	0·50	392	282	174	0·9	1·7	2·9
	55	0·63	16·12	83	194	13·20	29·32	1·04	0·70	0·84	494	370	239	2·1	1·9	3·5
Natural regeneration of *Pinus sylvestris*	11	0·01	0·16	28	6	0·04	0·20	0·04	0·04	0·04	17	17	7	2·4	2·4	5·7
	14	0	0·18	26	7	0·18	0·36	0·02	0·03	0·03	4	12	7	5·0	2·5	4·3
Plantation of *Pinus nigra*	31	0	17·37	51	341	5·44	22·81	0·67	1·00	1·47	156	136	113	4·3	7·4	13·0

on young trees, but in general no appreciable weight of cone is produced up to 14 years of age, after which the weight of cone per tree tends to increase considerably as the trees become older.

(*b*) Branches (Table II, Fig. 2)

The branches were separated into living and dead. Most of the branches initiated on the young trees survive for a number of years and dead branches do not form a significant proportion of the canopy weight until the trees attain 11 years of age. Afterwards mortality of the lower branches is considerable, and on the older trees living and dead branches occur in about equal amounts. The number, average weight, and total weight of living branches and the weight of dead branch material increase as the trees mature.

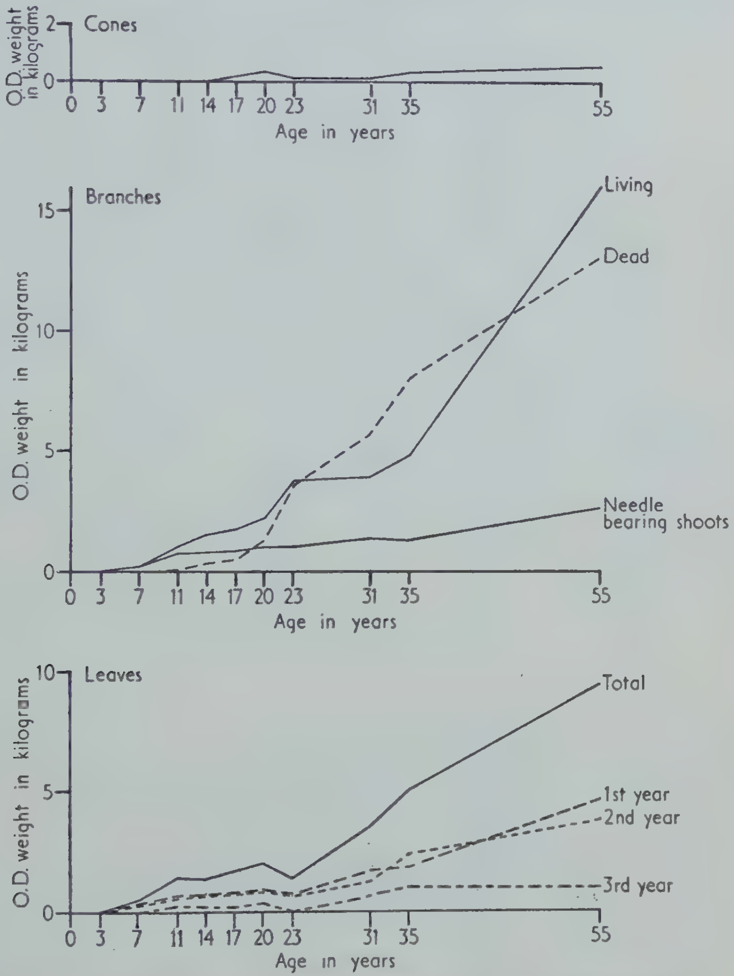

FIG. 2. Average canopy weights for sample trees of *Pinus sylvestris* in plantations

TABLE III

The Leaves of the Sample Trees

		Total wt. of leaves				No. of fascicles				Surface area of foliage						
	Age yrs.	1st yr. kg.	2nd yr. kg.	3rd yr. kg.	Total kg.	1st yr. (× 100)	2nd yr. (× 100)	3rd yr. (× 100)	Total (× 100)	1st yr. m.²	2nd yr. m.²	3rd yr. m.²	Total m.²	Av. no. fascicles per 1st yr. leaf-bearing shoot	Av. wt. leaf per 1st yr. leaf-bearing shoot g.	Wt. of 1st yr. leaves / Wt. of 1st yr. leaf-bearing shoots
Plantations of *Pinus sylvestris*	3	<0·01	<0·01	<0·01	<0·01	3	2	<1	5	—	—	—	—	—	—	—
	7	0·28	0·13	0·03	0·04	108	45	15	168	2·84	1·40	0·31	4·55	51	1·3	3·1
	11	0·60	0·55	0·22	1·37	300	233	102	635	6·15	5·77	2·21	14·13	59	1·2	2·6
	14	0·61	0·53	0·15	1·29	339	239	51	629	6·28	5·55	1·55	13·88	52	0·9	2·2
	17	0·70	0·68	0·21	1·59	284	231	98	613	7·20	7·07	2·18	16·45	36	0·9	3·2
	20	0·81	0·84	0·29	1·94	377	329	107	813	8·28	8·79	2·92	19·99	32	0·7	3·2
	23	0·72	0·65	0·02	1·39	415	479	6	900	7·40	6·80	0·23	14·43	19	0·3	4·8
	31	1·69	1·19	0·61	3·49	569	327	145	1,041	17·38	12·46	6·23	36·07	32	1·0	4·7
	35	1·83	2·40	0·87	5·20	1,039	1,010	383	2,432	18·75	25·01	9·88	53·64	27	0·5	5·1
	55	4·71	3·85	0·96	9·52	2,261	1,390	338	3,989	48·38	40·18	9·76	98·32	46	1·0	4·5
Natural regeneration of *Pinus sylvestris*	11	0·10	0·10	0·03	0·23	70	69	16	155	0·98	1·07	0·40	2·45	45	0·6	2·5
	14	0·07	0·07	0·02	0·16	56	38	5	99	0·75	0·70	0·25	1·70	140	1·8	3·5
Plantation of *Pinus nigra*	31	2·17	3·06	2·43	7·90*	427	364	335	1,150*	24·63	33·83	26·90	87·66*	27	1·4	3·3

* Inclusive of 4th yr. leaves.

Leaves are not carried along the whole length of a branch but are located on the distal shoots up to 4 years of age. The older trees compared with the younger ones form a greater weight and number of new shoots each year, but the average shoot weight is less than on the younger trees.

(*c*) Leaves (Table III, Fig. 2)

A pair of needles are carried in each fascicle of Scots pine. Occasionally leaves may persist up to 4 years of age, but 4-year-old needles were not collected since they are not present in any significant quantity and fall from the branches easily. They do not appear to be physiologically active and are sometimes covered with algae. The weights and numbers of 3-year-old needles are also proportionally small, the vast bulk of the leaf mass being composed of leaves in their first or second years in almost equal quantities. The weight of leaf carried on each tree initially increases rapidly, then becomes more or less constant in the thicket stage, i.e. from 11 to 23 years, and increases again with the advent of thinning. Fascicle number and area were also determined and gave a similar variation with tree age to that of weight. On the oldest tree there were nearly 400,000 fascicles with a total surface area of almost 100 square metres. In calculating leaf area it was assumed that the pair of leaves comprising a fascicle formed a cylinder when pressed together, and the surface area of each fascicle was calculated by the formula, leaf area $= \pi l \frac{1}{2}(d+d_1)+2dl$, where l is the fascicle length, d the width of the flat leaf surface, and d_1 the diameter of the two leaves at 90° to the flat surface.

The average number of leaves per distal shoot varies between nineteen and fifty-one, the variation showing no close relationship to tree age, although tending to decrease with increased age. Since the terminal shoots are on the average much smaller for the older trees, the leaf : leaf bearing shoot weight ratio becomes greater with increased tree age.

2. *Bole* (Table V, Fig. 3)

The boles increase in weight relatively slowly at first, but after the twentieth year the weight increment rises steeply. An increase in bole volume or weight is dependent upon either height or diameter growth, and although height growth is decreasing in the oldest plantations, the increase of bole girth at this stage is sufficient to maintain the weight increment. The bole increment must eventually fall off, but the age range of the investigated plantations is not sufficiently extensive to include examples of this. The relationship between age and bole weight for the sample trees may be expressed by the formula

$$\log_{10} W = -0{\cdot}24+3{\cdot}23 \log_{10} t$$

where $W =$ bole weight in grammes and t age in years.

3. *Total shoot* (Table V)

Rutter (1955) has investigated the relationships between the dry weight of the whole shoot inclusive of leaves, branches, and bole to the height and diameter of bole in young stands of *Pinus sylvestris* and obtained the following regressions:

$$\log_{10} W = -1{\cdot}34 + 1{\cdot}89 \log_{10} h$$

and

$$\log_{10} W = 1{\cdot}38 + 2{\cdot}41 \log_{10} d$$

where W = mean bole weight in grammes, d = mean hypocotyl diameter in centimetres, and h = mean height in centimetres.

TABLE IV

The Average Root Systems of Sample Trees

	Age yrs.	Tap-root kg.	Roots >0·5 cm. diam. kg.	Rootlets <0·5 cm. diam. kg.	Total root system kg.	Max. root depth cm.	Max. root length cm.
Plantations of *Pinus sylvestris*	3	0	0	0·002	0·002	0	0
	7	0·06	0·03	0·61	0·70	38	18
	11	0·45	0·37	1·79	2·51	88	65
	14	0·55	0·19	1·26	2·00	18	38
	17	0·85	0·41	1·00	2·26	64	105
	20	1·05	0·62	0·93	2·60	70	145
	23	3·92	1·45	2·34	7·71	140	100
	31	4·65	3·67	3·35	11·67	125	140
	35	11·17	7·25	5·07	23·49	140	200
	55	19·53	8·74	16·57	44·84	150	350
Natural regeneration of *Pinus sylvestris*	11	0	0·06	0·13	0·19	65	88
	14	0·06	0·18	0·31	0·55	140	100

TABLE V

The Average Weight of Sample Trees

	Age yrs.	Wt. of living canopy kg.	Wt. of bole kg.	Wt. of living shoot (i.e. canopy and bole) kg.	Wt. of root system kg.	Total wt. of living tree kg.	Wt. of living shoot / Wt. of root system
Plantations of *Pinus sylvestris*	3	0·003	0·002	0·005	0·002	0·007	3·6
	7	0·64	0·21	0·85	0·70	1·55	1·2
	11	2·39	1·24	3·63	2·51	6·14	1·4
	14	2·80	1·62	4·42	2·00	6·42	2·2
	17	3·39	2·88	6·27	2·26	8·53	2·8
	20	4·47	5·02	9·49	2·60	12·09	3·7
	23	5·30	12·18	17·48	7·71	25·19	2·3
	31	7·55	34·45	42·00	11·67	53·64	3·6
	35	10·66	52·28	62·94	23·49	86·43	2·7
	55	26·28	127·21	153·49	44·84	198·33	3·4
Natural regeneration of *Pinus sylvestris*	11	0·40	0·30	0·70	0·19	0·89	3·7
	14	0·34	0·89	1·23	0·55	1·78	2·2
Plantation of *Pinus nigra*	31	25·27	71·97	97·24	—	—	—

Similar linear relationships for the whole shoots between the logarithms of bole weight and diameter at base or height are found for these older plantations, the corresponding regression formulae being

$$\log_{10} W = -1{\cdot}23 + 1{\cdot}92 \log_{10} h$$

and

$$\log_{10} W = 1{\cdot}40 + 2{\cdot}64 \log_{10} d$$

4. *Root system* (Table IV, Fig. 4)

For convenience of sampling, the root system was divided into three arbitrary groups, (1) the main tap-root, (2) roots with a diameter greater than

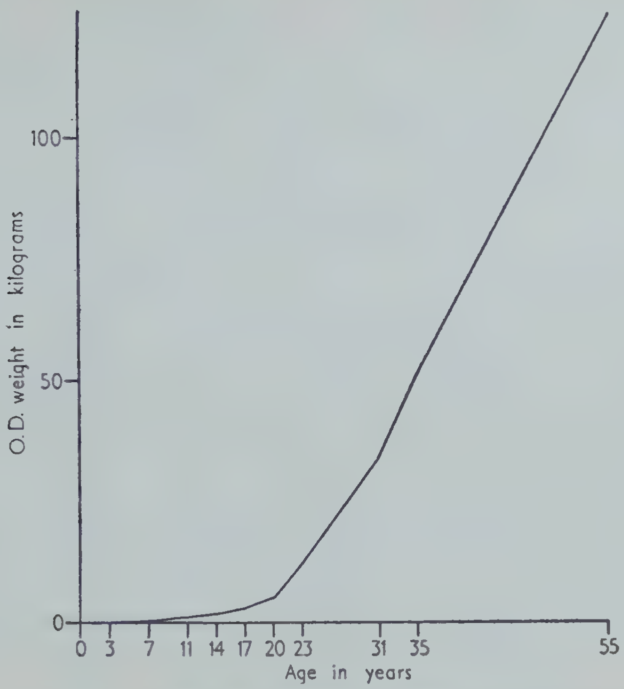

FIG. 3. The average bole weights for sample trees of *Pinus sylvestris* grown in plantations

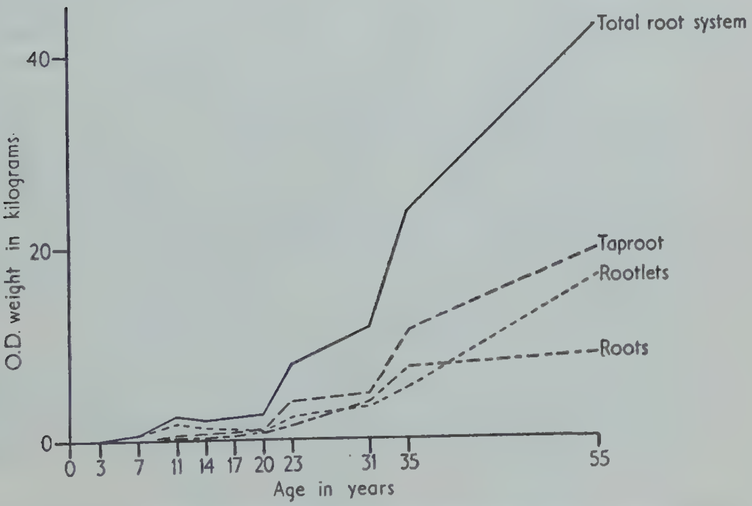

FIG. 4. The average weights for the root systems of sample trees of *Pinus sylvestris* grown in plantations

0·5 cm., and (3) terminal rootlets with a diameter of less than 0·5 cm. This nomenclature for the three root classes is used throughout this account.

The lengths of the lateral roots of the sample trees were measured. In sandy well-drained soils, such as those of the Breck, there is little restriction to rooting, and roots have readily penetrated downwards by the twenty-third year to reach the underlying boulder clay at a depth of about 1·5 m. in the sample plots. The boulder clay presents an effective barrier to root growth, and little farther downward penetration occurs. In contrast, the lateral spread of the roots, as indicated by the maximum root length for the sample trees, increases throughout the 55-year period. At first when the stocking density is more or less constant, the maximum lateral length of root for the sample trees increases rapidly with greater tree age, until by the twentieth year it almost equals the calculated mean distance between trees.[1] Afterwards, maximum root spread tends to be just less than the mean distance between trees and the two increase together as the trees become more widely spaced by successive thinnings.

The rooting zones of individual trees frequently overlap since the trees are not exactly uniformly distributed and the terminal rootlets may extend for considerable distances beyond the 0·5 cm. root-diameter limit. The weight of the root system increases considerably after the twentieth year, and by the fifty-fifth year the tree has a root system of almost 45 kg. dry weight, nearly half of which is tap-root.

5. *General proportions* (Tables V and VI, Fig. 5)

Expressed as percentages of the total weight of each tree, the relative proportions of the canopy, bole, and root system differ considerably for successive stages of development. The most significant change that occurs with greater age of the plantation is the increasing dominance of the main stem at the expense of both the canopy and root system. Initially, leaves represent a considerable part of the canopy, and indeed of the whole tree, but the percentage weight of the leaf mass decreases rapidly up to the twenty-third year when leaves and living branches occur in almost equal quantities. The decrease in the percentage of living branches is not so marked as that for leaves, since some branches continue to enlarge and attain a great size. The increasing weight of dead branches carried on the tree also compensates to some extent for the death of living branches, but at 20 years of age the proportion of dead branches also decreases as a result of pruning.

The root system of the 7-year-old trees are composed mainly of rootlets and account for almost half of the total plant weight, but as the trees mature the root systems represent a smaller proportion of the living trees. Associated with this decrease in the percentage weight of the root system as a whole, the

[1] The mean distance between trees was calculated by the formula,

$$\text{mean distance} = \sqrt{(1{\cdot}0746/\text{tree density})}$$

and for the plantations, arranged in order of increasing age from 7 to 55 years, is 155, 165, 149, 143, 146, 178, 221, 247, and 390 cm. respectively.

TABLE VI

The Weight Distribution of Average Sample Trees as Percentages of the Living Tree

	Age yrs.	Living branches	Leaves	Cones	Living canopy	Bole	Whole shoot	Rootlets	Roots	Tap-root	Whole root system	Dead branches
Plantations of *Pinus sylvestris*	3	14·6	35·0	0	49·6	28·3	77·9	22·1	0	0	22·1	0
	7	12·9	28·4	0	41·3	13·5	54·8	39·4	1·9	3·9	45·2	0
	11	16·6	22·3	0	38·9	20·2	59·1	29·2	4·4	7·3	40·9	0·2
	14	23·4	20·2	0	43·6	25·2	68·8	19·6	3·0	8·6	31·2	4·2
	17	19·6	18·6	1·5	39·7	33·8	73·5	11·7	4·8	10·0	26·5	5·3
	20	18·3	16·0	2·7	37·0	41·5	78·5	7·7	5·1	8·7	21·5	10·0
	23	15·1	5·5	0·4	21·0	48·4	69·4	9·3	5·8	15·5	30·6	14·2
	31	7·4	6·5	0·2	14·1	64·2	78·3	6·2	6·8	8·7	21·7	10·6
	35	5·9	6·0	0·4	12·3	60·5	72·8	5·9	8·4	12·9	27·2	9·3
	55	8·2	4·8	0·3	13·3	64·1	77·4	8·4	4·4	9·8	22·6	6·7
Natural regeneration of *Pinus sylvestris*	11	18·0	25·8	1·1	44·9	33·7	78·7	14·6	6·7	0	21·3	4·5
	14	10·1	9·0	0	19·1	50·0	69·1	17·4	10·1	3·4	30·9	10·1

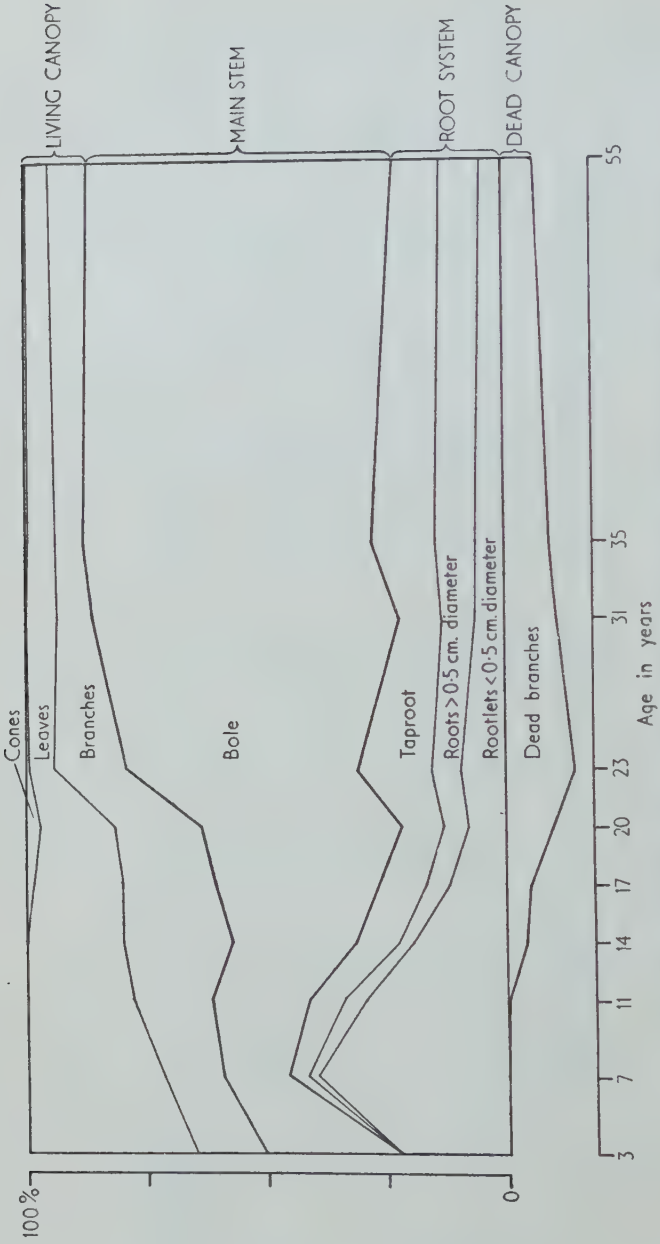

FIG. 5. The change with age in the relative proportions of the sample trees of *Pinus sylvestris* as percentages of the oven-dry weight of the living tree

proportion of the larger roots tends to increase so that the percentage weight of rootlets decreases rapidly.

For all the woodlands the weight of shoot is consistently greater than that of the associated root system, the shoot : root-system ratio having an average value of 2·7. The ratio tends to increase with greater age.

6. *Comparison of natural and planted trees of Scots pine* (Tables II–VI)

When the sample trees from the two areas of natural regeneration are compared with those of plantations of the same age, the greater luxuriance of the planted trees is obvious. The various subdivisions of the living canopy, bole, and root system of the planted trees are all heavier than the corresponding parts of natural trees. The intense root competition existing in the crowded stands of natural regeneration is indicated by the greater root depth and lateral spread, even though individually the root systems are of smaller weight than those of the corresponding planted trees. Competition apparently affects the tree proportions since the percentage weights of bole and root are greater in the natural stands.

7. *Comparison of* Pinus sylvestris *and* Pinus nigra (Tables II–VI)

The canopy and bole weights of the sample trees of Scots pine are much less than for Corsican pine of the same age at Thetford Chase. Although both trees have a similar number of living branches in the crown, the branches of *Pinus nigra* are larger with heavier leaf-bearing shoots. On leaf-bearing shoots of comparable age, Corsican pine has fewer fascicles than Scots pine, but the needles are larger and persist longer so that the total leaf mass per tree is much greater.

DRY-MATTER PRODUCTION BY AVERAGE TREES OF *PINUS SYLVESTRIS*

1. *Loss of dry matter*

The weight of a tree is not a complete measure of the total amount of organic matter produced during its lifetime since some natural loss occurs. This loss of plant material arises in two principal ways, (1) by processes such as respiration and (2) by the death of certain plant organs which may be shed naturally or removed by the forester.

Moller (1947) has measured the rate of respiration for stems of beech, ash, and spruce, and from the volume data of Oppermann (1912) has estimated the annual loss caused by respiration. Expressed as percentages of the dry matter of the woody portions above ground, the annual loss varied from 12·5 to 3·7 per cent., decreasing as the trees became taller. More assimilate was used for respiration by the two hardwoods than by the conifer. Since respiration is fundamental to the growth processes the use of assimilate during respiration does not represent a reduction of dry-matter production in the same way as the shedding of dead leaves, for instance.

The loss of dry matter by mortality varies considerably for different sections of a tree. Apart from outer bark relatively little is lost from the main stem, but in contrast the leaves are so short-lived that the leaf mass on a tree is composed of only part of the leaves produced during the 4-year period prior to sampling. Branches are intermediate between bole and leaves in this respect since they live longer than leaves and are not shed so readily, often when dead remaining attached to the bole. From observations of the root system there appears to be relatively little loss of dry matter from actively growing trees by the mortality of the large tap and lateral roots, but undoubtedly considerable decomposition of rootlets occurs. Orlov (1955) has suggested that in a 25-year-old spruce forest the annual decomposition of feeding roots corresponds to about half the weight of the leaf fall, and in a 50-year-old spruce forest the corresponding fraction is one-fifth.

2. *Gross production* (Tables VII and VIII, Fig. 6)

Since the trees of Scots pine form an age series it has been possible to calculate the loss of dry matter by mortality for the various components of the tree crowns and so determine the aggregate production at different stages of development. This loss is accumulative, increasing with age, and for the 55-year-old tree the overall production of cones, leaves, and branches exceeds the weights carried on the tree by about 22, 10, and 2 times respectively, the gross production of aerial shoot being about twice its weight.

TABLE VII

Total Weight of Dry Matter Produced by Average Sample Trees

	After	Cones kg.	Branches kg.	Leaves kg.	Bole kg.	Whole shoot kg.
Plantations of *Pinus sylvestris*	3 yrs.	0	0·001	0·002	0·002	0·005
	7 yrs.	0	0·21	0·56	0·21	0·98
	11 yrs.	0	1·26	2·32	1·24	4·82
	14 yrs.	0·02	1·84	4·14	1·62	7·62
	17 yrs.	0·23	3·68	6·10	2·88	12·89
	20 yrs.	0·90	5·13	8·37	5·02	19·42
	23 yrs.	1·55	11·79	10·66	12·18	36·18
	31 yrs.	2·35	18·59	20·30	34·45	75·69
	35 yrs.	3·29	26·43	27·34	52·28	109·34
	55 yrs.	13·39	34·11	92·74	127·21	267·45

TABLE VIII

The Average Dry-matter Production of Sample Trees as Percentages of Weight of Shoot Produced

	Age yrs.	Cones	Branches	Leaves	Bole
Plantations of *Pinus sylvestris*	3	0	20·0	40·0	40·0
	7	0	21·4	57·2	21·4
	11	0	26·2	48·1	25·7
	14	0·3	24·1	54·3	21·3
	17	1·8	28·6	47·3	22·3
	20	4·6	26·4	43·1	25·9
	23	4·3	32·6	29·4	33·7
	31	3·1	24·6	26·8	45·5
	35	3·0	24·2	25·0	47·8
	55	5·0	12·7	34·7	47·6

Because of the differential loss of dry matter the relative proportions of the accumulated weights of cones, branches, leaves, and bole differ considerably from those of the living tree. More dry matter is contained in the boles than in the living crowns of all the sample trees of 20 years of age and over, but this dominance of bole is greatly reduced when the figures are expressed as gross production. At no stage is the total production of bole greater than that of crown. Cone and leaf formation in particular both represent a more significant proportion of total production, although the percentage production of leaf still declines with greater age of the tree.

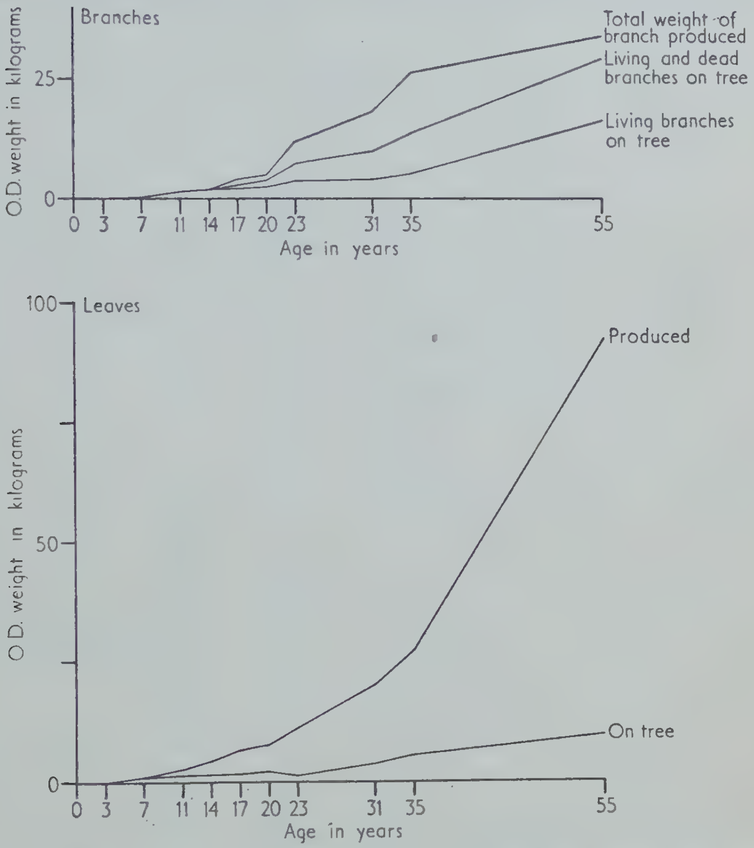

FIG. 6. The difference for branches and leaves in the weights produced and carried on the average sample trees of *Pinus sylvestris* grown in plantations

DRY-MATTER CONTENT OF WOODLANDS

1. *Plantations of* Pinus sylvestris (Tables IX and X, Fig. 7)

An estimate of the total amount of dry matter contained in the trees of each forest stand was obtained by multiplying the average tree weight by the stocking density.

The weight of bole present per hectare of woodland increases slowly up to the seventh year, then afterwards more rapidly to reach a maximum at 35 years of age. The relatively low weight of bole contained in the oldest plantations results primarily from its poor height growth although, as noted previously, individual bole weights continue to become heavier with greater age.

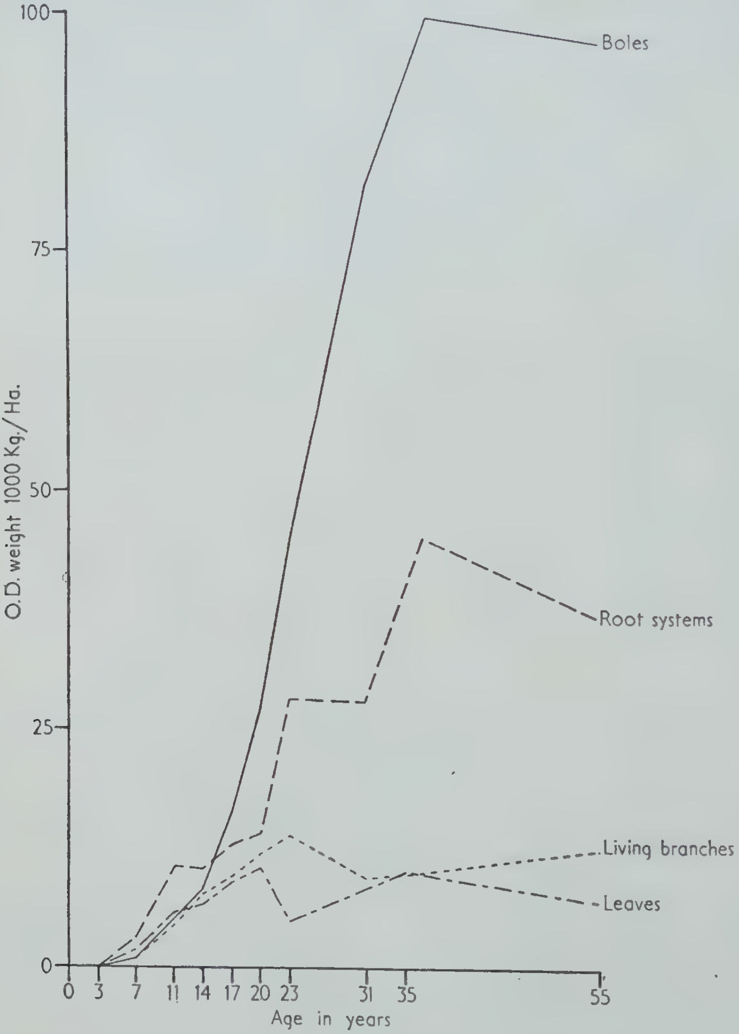

FIG. 7. The weights of the plantations of *Pinus sylvestris*

It appears from volume tables that if the oldest plantation had been of the same quality class as the younger plantations the increment in bole weight would have been maintained.

Unlike the boles, the leaves and branches after passing through an initial build-up period tend to reach a more or less constant maximum weight by

TABLE IX

The Total Dry Weights (1,000 kg./ha.) of the Woodlands

	Age	Leaves				Leaf	No. of					Whole				Root	
	yrs.	1st yr.	2nd yr.	3rd yr.	Total	area ha./ha.	fascicles ($\times 10^6$)	Cones	Living branches	Dead branches	Boles	living shoot	Tap-roots	Roots	Rootlets	system	Total
Plantations of *Pinus sylvestris*	3	<0·01	<0·01	<0·01	0·01	—	3	0	0·01	0	0·01	0·03	□	□	0·01	0·01	0·04
	7	1·35	0·63	0·14	2·12	2·19	81	0	0·96	0	1·01	4·09	0·29	0·14	2·93	3·37	7·46
	11	2·54	2·33	0·93	5·80	5·98	269	0	4·31	0·04	5·25	15·36	1·90	1·14	7·57	10·62	25·98
	14	3·16	2·75	0·78	6·69	6·94	326	0·05	7·79	1·40	8·41	22·93	2·85	0·99	6·54	10·38	33·31
	17	3·95	3·84	1·18	8·97	9·28	346	0·73	9·42	2·54	16·24	35·36	4·79	2·31	5·64	12·75	48·11
	20	4·37	4·54	1·57	10·48	10·79	439	1·73	11·99	6·53	27·11	51·31	5·67	3·35	5·02	14·04	65·35
	23	2·62	2·37	0·07	5·06	5·25	328	0·40	13·83	13·03	44·34	63·63	14·27	5·28	8·52	28·07	91·70
	31	4·01	2·82	1·45	8·28	8·55	247	0·21	9·41	13·44	81·65	99·55	11·02	8·70	7·93	27·65	127·20
	35	3·46	4·54	1·83	9·83	10·14	460	0·72	9·60	15·25	98·81	118·96	21·11	13·70	9·58	44·40	163·36
	55	3·58	2·93	0·73	7·24	7·47	303	0·48	12·25	10·03	96·68	116·65	14·84	6·64	12·59	34·07	150·72
Natural regeneration of *Pinus sylvestris*	11	5·80	5·80	1·74	13·34	14·21	899	0·58	9·28	2·32	17·40	40·61	□	3·48	7·54	11·02	51·63
	14	1·94	1·94	0·56	4·44	4·72	275	0	5·00	5·00	24·70	34·13	1·67	5·00	8·60	15·26	49·39
Plantation of *Pinus nigra*	31	3·65	5·14	4·08	13·27*	14·73*	193*	0	29·18	9·14	120·91	163·36	Not sampled				

* Inclusive of 4th yr. leaves.

about the twentieth year, although there is some indication of a gradual decrease in the weight of leaf and branch material in the older plantations. No tree had been extracted from the thinned woodlands for several years prior to sampling so that their canopies were in a relatively stable condition with few gaps between the tree crowns. The constancy of leaf weight in close woodlands has been reported for a number of tree species and is regarded as indicative of the attainment of the full utilization of incident light by a forest under the existing ecological and sylvicultural conditions.

TABLE X

The Distribution of Rootlets in the Soil Volume

		Wt. rootlets per litre of soil volume at depths			
	Age yrs.	0–12·5 cm. g.	12·5–25·0 cm. g.	25·0–37·5 cm. g.	37·5–50·0 cm. g.
Plantations of *Pinus sylvestris*	7	0·52	0·66	0·82	0·36
	11	0·35	1·13	1·89	2·69
	14	2·07	1·22	0·92	1·01
	17	1·44	1·18	1·11	0·76
	20	1·55	0·91	1·02	0·56
	23	2·10	2·32	1·08	1·31
	31	1·47	1·91	1·80	1·16
	35	2·14	2·60	1·63	1·28
	55	4·88	1·62	2·21	1·36
Natural regeneration of *Pinus sylvestris*	11	2·48	2·37	0·56	0·71
	14	2·03	2·18	2·14	0·58

For obvious reasons the subterranean parts of a woodland are more difficult to sample accurately than the aerial shoots, and some of the variability in the weights of the root systems reflects this. Nevertheless for the plantations up to 35 years of age the root systems tend to increase in weight as the plantations become older. The decrease in the total weight of the root system in the oldest woodland parallels that of boles and results mainly from a decrease in the tap and lateral root weights. It is interesting that the decrease does not occur for the small rootlets. The rootlets are largely concentrated in the upper soil layers, and as the plantations mature the frequency of rootlets is intensified in the surface soil as well as down the rest of the soil profile. Root growth appears to be very active and any gaps created by thinning are soon invaded by the root systems of the surviving trees.

2. *Comparison of planted with natural regeneration of* Pinus sylvestris (Table IX)

The average trees of the natural regeneration are smaller than those of similar age grown in plantations, but the greater number of trees in the areas of natural regeneration reduces the difference when expressed on a weight and area basis. When the two 11-year-old tree stands are compared, the area of natural regeneration contains the larger weight of roots, boles, living branches, and leaves and the greater number and area of leaves. Although mortality has been high in the 14-year-old stand of natural regeneration, the total weight of living material is still greater than in comparable planted woodlands.

3. *Comparison of plantations of* Pinus sylvestris *and* P. nigra (Table IX)

Although the stocking density of the plantation of Corsican pine is lower than that of the Scots pine stand of comparable age the average trees of *Pinus nigra* are individually much larger so that the total weight of leaves, living branches, and boles present in the stand of Corsican pine is much greater than in the Scots pine plantation. Unfortunately only the rootlets of the Corsican pine root system were sampled, but the total weight of rootlets per hectare of woodland is considerably less than in the stand of *Pinus sylvestris*.

DRY-MATTER PRODUCTION BY PLANTATIONS OF *PINUS SYLVESTRIS*

1. *Thinnings* (Table XI)

Harvesting of the forest crop begins when the plantations are between 20 and 23 years of age and is continued for the rest of their life. On an average the total weight of trees felled per annum in an hectare of woodland is about 8,000 kg. of dry matter, of which approximately half is bole material. In practice, thinning is not carried out every year, but on a rotation usually of 4 years or more. Normally only the tree boles are extracted, the crowns and root systems being left to decompose in the plantation. By careful, selective thinning the forester aims at maintaing a regular distribution of the remaining

TABLE XI

Weight (1,000 kg./ha.) of Trees Removed as Thinnings from Plantation of Pinus sylvestris

Period yrs.	No. of trees removed	Cones	Living branches	Leaves	Boles	Root systems	Total	Average/ annum
20–23	1,760	0·4	5·3	2·9	15·1	9·1	32·8	10·9
23–31	1,270	0·1	4·9	3·1	29·6	12·3	50·0	6·3
31–35	480	0·1	2·2	2·1	20·8	8·4	33·6	8·2
35–55	1,130	0·6	12·0	8·3	101·4	38·6	160·9	8·0

TABLE XII

Total Weight of Dry Matter (1,000 kg./ha.) Produced by Plantations of Pinus sylvestris *(inclusive of Thinnings and Canopy Loss)*

Age yrs.	Cones	Branches	Leaves	Boles	Root system	Total
5	0	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
7	0	1·0	2·7	1·0	3·4	8·1
11	0	5·3	9·8	5·2	10·6	30·9
14	0·1	9·6	21·5	8·4	10·4	50·0
17	1·3	20·8	34·4	16·2	12·7	85·4
20	4·9	27·7	45·2	27·1	14·0	118·9
23	7·8	57·8	55·5	59·4	37·2	217·7
31	10·3	78·3	84·4	126·3	49·1	348·4
35	12·3	95·0	99·4	164·3	74·2	445·2
55	25·6	105·1	186·1	263·6	102·5	682·9

stock and tries to favour the more desirable trees so that the thinnings tend to consist of a higher proportion of badly formed trees than the remaining stock.

2. *Gross production* (Table XII, Figs. 8 and 9)

Before the advent of thinning the gross production of the plantations can be estimated by multiplying the total weight of dry matter formed by the average trees by the stocking densities. When thinning begins, the gross production of the plantation consists of that of both the stock and the removed trees. The increments of the plantations were determined from smoothed-out graphs of plantation production but do not include an allowance for rootlet mortality since this could not be determined accurately. It seems from Orlov (1955) that the annual loss of rootlets may amount to about 5 per cent. of the total production.

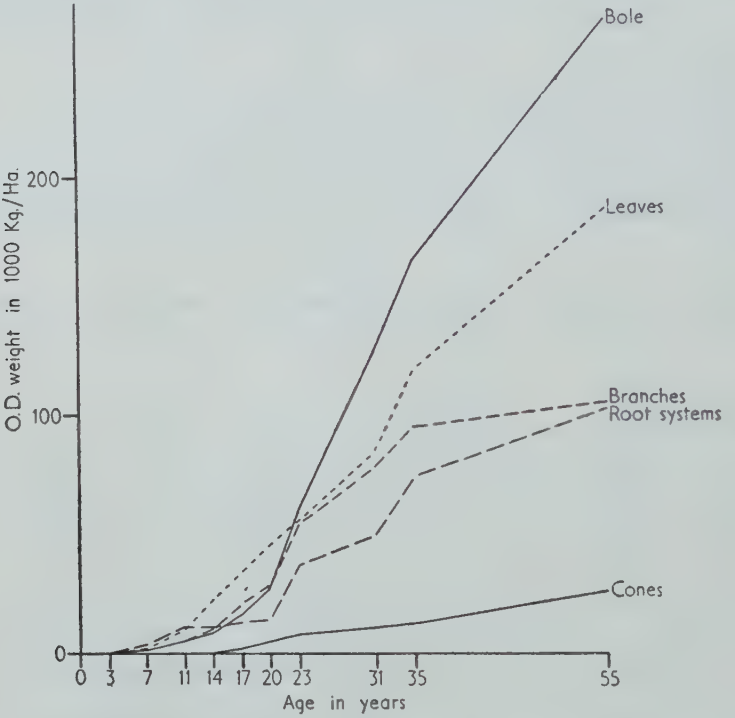

Fig. 8. The gross production of dry matter by plantations of *Pinus sylvestris*

The canopy comprising cones, branches, and leaves accounts for almost half of the dry matter produced and the remainder is distributed broadly in the ratio of 2:1 between the boles and root systems respectively. After 55 years, almost 700,000 kg. of dry matter have been produced per hectare by the oldest plantation. Since the younger plantations are of a slightly better quality class it seems likely that their total production will be greater when they reach this age.

After 20 years, a linear relationship exists between plantation age and the total weights of cones and leaves produced, so that the current annual increment per hectare becomes constant. The current annual increments of branches, boles, and root systems do not reach a constant value but increase to a maximum between 23 and 29 years of age and then decrease. The maximum current increment of dry matter per annum by the plantations is attained by the twenty-eighth year.

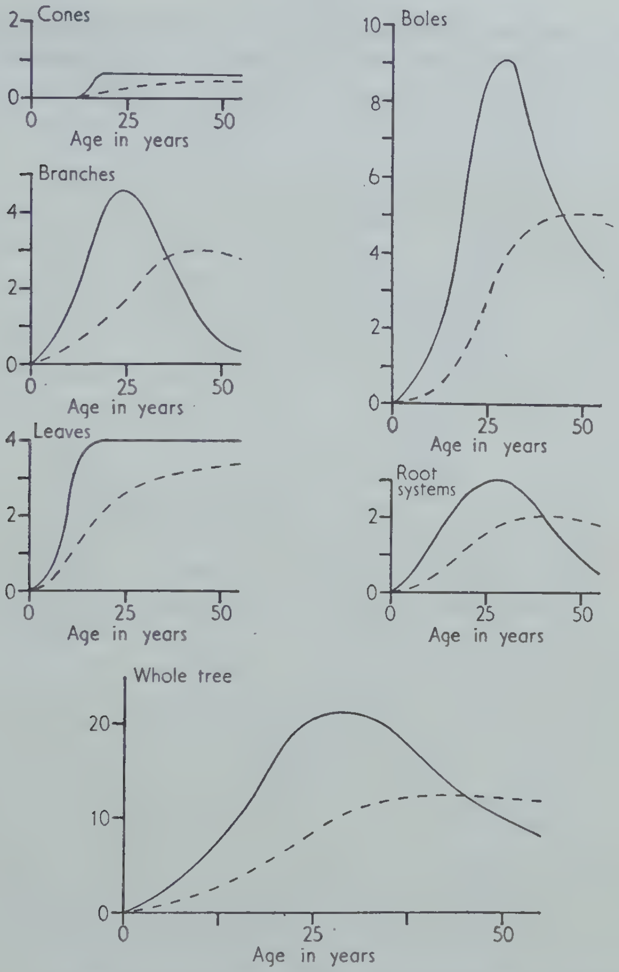

FIG. 9. Current (solid line) and mean (broken line) annual increments of dry matter (kg./ha.) by plantations of *Pinus sylvestris*

The mean annual increments all increase to a maximum value and, except for cones and leaves, decline in the older plantations. The maximum mean rate of accumulation of dry matter per hectare is attained by about the forty-third year, but this is rather earlier than would be expected from the volume tables for Quality Class I plantations and results from the poor height growth of the oldest stand.

DISCUSSION

Despite the economic importance of timber, surprisingly little is known of the basic plant production of woodlands as compared with some other ecosystems. The investigations of marine biologists such as Riley, Stommel, and Bumpus (1949) in the North Atlantic and Steele (1956) in the North Sea have resulted in a much more detailed appreciation of the quantitative aspects of production by phytoplankton in the waters around Great Britain than is available for timber production in British woodlands. Some intensive studies, notably by Burger (1939 to 1948), have been made in European forests, but from comparisons of yield tables it seems that the rate of tree growth in Great Britain differs from that of continental Europe (Lehtpere, 1956), in general being superior for the British Isles.

This account is primarily concerned with the dry-matter production by individual trees and by forest stands of *Pinus sylvestris* grown in the extensive forests of Thetford Chase (Macdonald, 1939). The plantation weights have been calculated from the stocking density and the dry weights of sample trees having average dimensions of bole and canopy. Because of the possible variability between these dimensions and dry weight, the determination of plantation weight in this way may lead to an underestimate, although Güde (1955) has found for spruce that the mean tree is truly representative of a plantation when both stem and crown class are taken into account.

The current annual increment of dry matter is not constant and the efficiency of the plantations to utilize site factors for dry-matter production varies considerably. The maximum current annual increment is approximately 22,000 kg./ha., but the maximum mean annual increment, which includes the juvenile stages of plantation development, is much less, being only about 13,000 kg./ha. In the younger plantations the tree canopies and root systems do not completely occupy the available crown and root space, and the plantation increment is less than in areas of natural regeneration where there are many more trees. It seems probable that uneven-aged forests over a period of time would have a greater increment than the even-aged plantations since they would not pass through an initial phase with only small trees present to give a partial utilization of the site factors. Begley (1955) records an annual yield of approximately 5,400 kg./ha. of woody shoot material for 16-year-old chestnut coppice. The coppice system from the point of view of production has the advantage that the seedling stage is avoided and the shoots are produced from existing stools.

The maximum current or mean annual increments of the Scots pine plantations are greater than the annual yield for agricultural crops grown in the locality. Only a small proportion of the total dry matter produced by woodlands is, however, of commercial value and a considerable quantity of dry matter accumulates over the soil (Ovington, 1954). There are about equal areas of arable and grassland in Breckland, and of the arable land over half is devoted to cereals. Wheat and barley gave the highest and lowest yield figures that are available for cereals with 5,200 and 3,500 kg./ha. respectively, inclusive of both grain and straw. A heavier yield is obtained from root crops; sugar-beet, carrots, and mangolds inclusive of tops have annual dry-matter yields of 9,000, 6,500, and 6,000 kg./ha. respectively. Hay production from a hectare of grassland is about 7,500 kg./ha./annum. The relatively high annual yields, despite the lack of intensive cultivation, by the Scots pine plantations probably reflect the ability of a forest crop to carry a considerable weight of leaf for photosynthesis and to develop a root system which penetrates throughout the whole soil volume. In addition evergreen conifers are able to assimilate whenever conditions are suitable throughout the year (Freeland, 1944), whilst the growing period of most agricultural crops is limited. It is significant that the leaf weight carried by the plantations tends to become more or less constant and the ground vegetation is less luxuriant in this close canopy stage when light intensity at ground level is greatly reduced (Ovington and Madgwick, 1955).

TABLE XIII

Dry-matter Production, Leaf Area, and Weight of Rootlets in Plantations of Pinus sylvestris

Period yrs.	Wt. bole produced/annum/ sq.m. leaf surface kg.	Wt. dry matter produced/ annum/sq.m. leaf surface kg.	Wt. bole produced/annum/ kg. rootlets kg.	Wt. dry matter produced/ annum/kg. rootlets kg.	Relative growth rate of sample trees g./g./annum	Relative growth rate of plantations g./g./annum
3–7	0·02	0·18	0·16	1·35	1·37	1·33
7–11	0·03	0·15	0·22	1·18	0·37	0·34
11–14	0·01	0·05	0·08	0·50	0·09	0·16
14–17	0·03	0·12	0·37	1·63	0·15	0·18
17–20	0·04	0·13	0·73	2·36	0·13	0·11
20–23	0·14	0·42	1·46	4·45	0·23	0·20
23–31	0·11	0·22	0·98	1·91	0·09	0·59
31–35	0·10	0·25	1·06	2·70	0·11	0·62
35–55	0·05	0·12	0·35	0·83	0·04	0·21

The tree stems which constitute the major part of the forest crop only represent a very small proportion of the total dry matter produced, and the distribution of dry weight between the different plant organs varies considerably as the trees mature. Because of the regular shedding of branches, leaves, and cones from the trees and the extensive harvesting period, the stock of trees does not represent total dry-matter production and is not truly comparable to the stock of herbaceous plants. However, it has proved possible to determine the total weight of dry matter produced by the sample trees and plantations of *Pinus sylvestris* and to calculate the relative growth-rates as defined by Blackman and co-workers (1947 to 1955). The relative growth-rates of the trees (Table XIII) tend to be less than for herbaceous plants, but the growth of the trees is calculated throughout the whole year and not only during the period of active growth. Significant differences occur in leaf and rootlet efficiencies as measured by dry-matter production before and during the thinning periods. In the close-canopy plantations prior to thinning, relatively little dry matter is produced per unit weight or area of leaf, and it appears that many leaves are shaded and not assimilating actively. Thinning reduces the proportion of shade leaves so that after 20 years more dry matter is produced per unit weight of leaf. The intense root competition existing in forest stands has been demonstrated by trenching experiments (Korstian and Coile, 1938), and numerous studies have been made of root distribution in forest soils notably by Laitakari (1929) in Finland and more recently in Great Britain by Yeatman (1955). Compared with observations on root distribution relatively few data are available of root weights, but it is evident that in these plantations of *Pinus sylvestris* there is a rapid increase in the total weight of smaller rootlets, particularly in the surface soil horizons. The shoot:root ratio is consistently greater than unity.

Only one plantation of *Pinus nigra* was examined in the sampling area for comparison with Scots pine, and its production is much greater than that of *Pinus sylvestris*. The greater production is associated with an increased weight of leaf, but fewer rootlets are present in the Corsican plantation.

SUMMARY

1. In nine plantations and two stands of natural regeneration of *Pinus sylvestris* and one plantation of *Pinus nigra* detailed measurements of the tree boles and crowns have been made and average trees selected for dry-weight studies.

2. The oven-dry weights of cones, branches, leaves, boles, and root systems of the sample trees have been determined and at 55 years of age the average tree of *Pinus sylvestris* weighs almost 200 kg. and carries just under 8×10^5 needles with a surface area of nearly 100 m.^{2}

3. The relative proportions of dry matter contained in the crown, bole, and root system vary considerably and, with greater age of the trees, the bole represents an increasing proportion of the tree weight.

4. The total weight of dry matter produced by a sample tree within its lifetime has been calculated and is much greater than the standing weight. The largest proportion of the total dry matter produced is contained in the crown and the least in the root system.

5. Except for the oldest plantation of *Pinus sylvestris* the weight of dry matter contained in the bole and root system of the stock increases with age, but the total weights of the leaves and living branches tend to remain more or less constant after an initial build-up period.

6. The total dry weights of boles, leaves, branches, roots, and cones produced by the plantations increases with greater age, and by 55 years of age the trees have produced almost 7×10^5 kg. of dry matter per hectare.

7. Current and mean increments of dry matter have been determined for the different components of the plantations, and the maximum mean incre-

ment of dry matter by the plantations is attained by about the forty-third year after planting.

8. In the young close plantations 1 kg. dry weight of needles annually produces from 0·6 to 1·9 kg. of dry matter, but in the thinned plantations, after 20 years of age, leaf efficiency increases and the comparable annual production is 1·2 to 4·4.

9. Comparisons are made between plantations and natural regeneration of *Pinus sylvestris.*

10. The dry weights of trees and plantations of *Pinus sylvestris* and *Pinus nigra* of identical age are compared and in general Corsican pine is more productive than Scots pine.

11. Attention is drawn to the relatively high productivity of *Pinus sylvestris* in the locality compared with agricultural crops.

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A Bivariate Test for the Clumping of Supposedly Random Individuals

BY

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With three Figures in the Text

ABSTRACT

A method is proposed for the analysis of plant distributions from the relative positions of individual plants rather than from the numbers of plants occurring in successive small areas. The method is illustrated by reference to the detailed distribution in nature of *Solidago rigida* and of plants infected with nettlehead, a virus disease of hops.

1. Data concerning the distribution of plants (or individuals) over an area are often examined by analysing counts of the numbers of plants that occur in successive areas of some fixed unit-size. Such tests are usually adequate for their purpose but they are not entirely satisfactory in that they disregard information concerning the distribution of plants within the unit areas and also depend on the method used for subdivision into unit areas. A fresh aspect of this examination (statistically a test for randomness) is put forward in this present note. We envisage two different situations. Firstly, it is assumed that the position of every plant of a given species in a wild community is known. This will be the case when a comprehensive plot is made of the community. We suggest a test of the randomness of dispersal of the plants using the coordinates of the position of each. Secondly, a square (or rectangular) lattice trial layout is supposed with plants (or trees) set at the lattice intersections. Some of the trial plants are found to be diseased, and it is desired to know whether these plants are randomly dispersed among the sound ones. In both cases the alternative to randomness is that there is clumping together of plants (or individuals) or that the distribution is more uniform than might be expected.

2. We suppose that there are N plants distributed over a square (or rectangular) area, A. Two adjacent sides of the square can be taken as the axes of cartesian coordinates and by dividing the coordinates of each plant by the length of the appropriate side of A we may discuss the dispersal within a unit square. Assume therefore that

$$(x_1, y_1), (x_2, y_2), \dots (x_N, y_N)$$

are the coordinates of the individual plants where no x or y is less than zero or greater than unity. Divide the square into k strips by lines parallel to the y-axis so that the ith strip contains n_i plants, $(i = 1, 2, \dots k)$. Obviously

$$\sum_{i=1}^{k} n_i = N.$$

The strips need not necessarily be of equal width. Under the hypothesis of

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randomness over the whole unit square A there is equal chance for observations to lie above or below the half-way line $y = \frac{1}{2}$. This same remark is true for any one of the strips. It will not be true if the plants tend to be clumped together.

Let y_{ij} be the y-coordinate of the jth plant in the ith strip, and let $\bar{y}_i$ be the mean of y in the ith strip. The total variation of the y's about $y = \frac{1}{2}$ can be split up into the variation of the strip means about $\frac{1}{2}$ and the variation of the y's about the strip means. Thus

$$\sum_{i=1}^{k}\sum_{j=1}^{n_i}(y_{ij}-\tfrac{1}{2})^2 = \underbrace{\sum_{i=1}^{k}\sum_{j=1}^{n_i}(\bar{y}_{ij}-\bar{y}_i)^2}_{\alpha}+\underbrace{\sum_{i=1}^{k}n_i(y_i-\tfrac{1}{2})^2}_{\beta}.$$

In repeated experimentation it may be shown that the average value of these quantities is

$$N\sigma_y^2 = (N-k)\sigma_y^2+k\sigma_y^2$$

where σ_y^2 is the square of the standard deviation of the distribution of y. If the hypothesis of randomness is tenable we shall expect therefore that the ratio β/α will be somewhere near $k/(N-k)$. If instead of randomness the observations tend to occur in clumps then the variation of the plants about the strip means will tend to be small compared with the variation of the strip means about $\frac{1}{2}$. A small value of β/α will indicate that the reverse of clumping is present, i.e. that the plants are too uniformly scattered over the unit area. The position is illustrated in Fig. 1 where (*a*) gives an example of clumping, (*b*) of regularity, and (*c*) of a random spread.

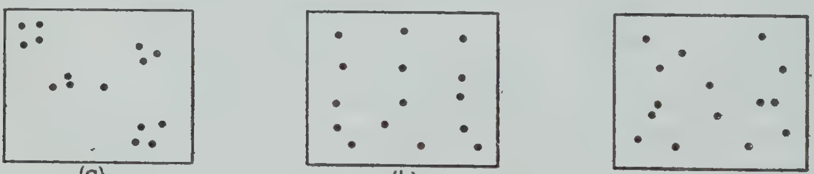
(a) (b) (c)

FIG. 1

3. For technical reasons instead of the simple ratio β/α we calculate as our test criterion

$$Z = 1{\cdot}151293 \log_{10}\left\{\frac{N(N-k)}{k^2}\,\frac{\beta}{\alpha}\right\}.$$

Tables I and II give the upper 5 per cent. and 1 per cent. levels of the distribution of Z when the numbers in the strips are all equal, i.e. $n_i = N/k$ for each strip. These tables can be used to determine whether or not a calculated value of Z is larger than might be expected on the null hypothesis of randomness.

Three points call for comment regarding the test. First consider k, the number of strips. We have not specified what this should be, and we do not propose to discuss it exhaustively here. We note, however, that the test will be most discriminating when each strip contains just one cluster, which suggests taking k equal to the total number divided by the cluster size. Often we do not know the cluster size but this will usually be small. In the absence of such information we suggest taking two points only to a group and, if N be odd, three in the last group.

This last leads on to a second point. Where the division into strips is at choice the numbers in the strips should be as nearly equal as possible. There will be occasions when this will not be possible, for example, when we consider diseased plants in a fixed layout. In this case we make the divisions so that no strip contains more (or less) than twice the average value, N/k. We calculate the variance of these numbers about the average, say

TABLE I

5 per cent. Significance points for Z

n_i \ k	4	5	6	7	8	9	10
2	1·0013	0·8599	0·7647	0·6945	0·6410	0·5980	0·5616
3	0·7167	0·6296	0·5714	0·5258	0·4903	0·4606	0·4360
4	0·6230	0·5549	0·5059	0·4680	0·4388	0·4134	0·3925
5	0·5762	0·5163	0·4729	0·4387	0·4123	0·3893	0·3703
6	0·5477	0·4928	0·4528	0·4210	0·3966	0·3745	0·3555
7	0·5287	0·4773	0·4394	0·4090	0·3859	0·3647	0·3458
8	0·5127	0·4660	0·4297	0·4004	0·3781	0·3575	0·3428

TABLE II

1 per cent. Significance points for Z

n_i \ k	4	5	6	7	8	9	10
2	1·4757	1·2202	1·0936	1·0114	0·9305	0·8631	0·8199
3	0·9886	0·8969	0·7963	0·7343	0·6860	0·6441	0·6118
4	0·8432	0·7586	0·6977	0·6461	0·6075	0·5732	0·5453
5	0·7750	0·7023	0·6474	0·6033	0·5698	0·5382	0·5119
6	0·7362	0·6703	0·6193	0·5781	0·5462	0·5174	0·4929
7	0·7110	0·6486	0·5986	0·5615	0·5313	0·5038	0·4799
8	0·6936	0·6349	0·5877	0·5500	0·5208	0·4939	0·4711

$n_i = N/k$

$$W = \frac{1}{k}\sum_{i=1}^{k}\left(n_i - \frac{N}{k}\right)^2.$$

The correction for unequal grouping to be added to the ϵ per cent. point of Z is

$$-\frac{CW}{\sqrt{N}}\left(\frac{k^5}{N^2\,(N-k)^3}\right)^{\frac{1}{2}}$$

where $C = 0{\cdot}349$ when $\epsilon = 5$ and $0{\cdot}493$ when $\epsilon = 1$. It will be noticed that this correction is negative which means that if the ϵ per cent. points given in Tables I and II are used for unequal numbers the test will in fact be more stringent than the nominal value implies. This is a satisfactory state of affairs. For values of N and k outside Table I, tables of the normal variance ratio Z may be used, the table being entered with $N-k$, k, and the Z calculated as before.

The third remark which one must make with regard to this Z test is that so far we have tested for randomness in the direction of y only. If significance has not been established in the y-strips the test should be repeated by drawing strips parallel to the x-axis and calculating the variation of the x's from their strip means and so on.

4. In this section we illustrate the application of our proposed criterion to test for randomness in wild communities. Cain and Evans (1952) give a map of about 2 acres of grassland in Livingston County, Michigan. In Fig. 2 we reproduce a portion of area approximately 90×80 ft. showing the plots of 48 individual plants of *Solidago rigida*. Reducing the coordinates of each plant to the unit square as reference we have the following points, arranged in order of ascending magnitude of the values of x.

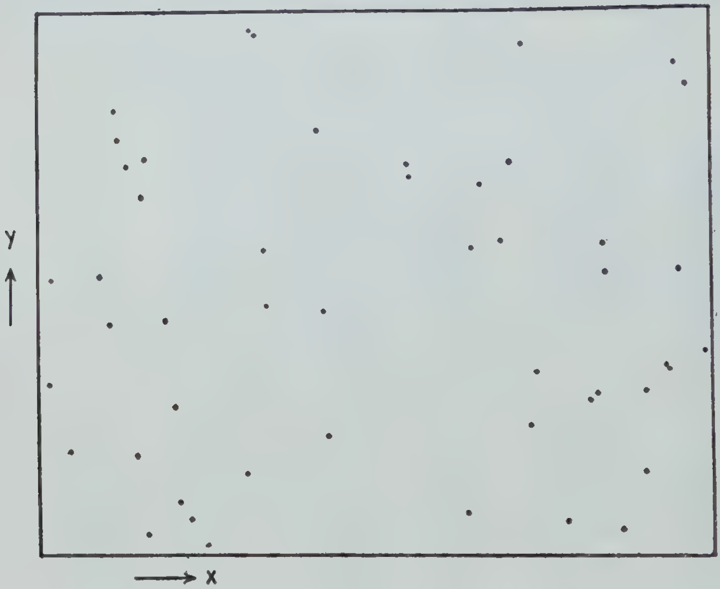

FIG. 2

If we decide to take the number in a group (or strip) equal to 2 then we

TABLE III

Coordinates of individual plants of Solidago rigida

x	y	x	y	x	y	x	y
0·017	0·149	0·191	0·446	0·554	0·570	0·836	0·096
0·021	0·692	0·207	0·430	0·556	0·281	0·843	0·939
0·050	0·658	0·214	0·818	0·643	0·421	0·846	0·561
0·091	0·075	0·229	0·509	0·650	0·719	0·871	0·505
0·109	0·781	0·314	0·763	0·661	0·061	0·903	0·711
0·114	0·342	0·320	0·372	0·691	0·237	0·907	0·067
0·121	0·153	0·329	0·860	0·706	0·518	0·934	0·558
0·131	0·295	0·341	0·575	0·723	0·477	0·937	0·954
0·149	0·182	0·343	0·716	0·736	0·035	0·949	0·904
0·157	0·333	0·421	0·298	0·743	0·965	0·953	0·679
0·163	0·049	0·429	0·214	0·790	0·189	0·964	0·339
0·164	0·312	0·435	0·728	0·826	0·272	0·993	0·456

may pair the y's and calculate the sum of squares of the deviations from $\frac{1}{2}$ and the sum of the squares of deviations from the strip means.

(For two in a group we have

$$\sum_{j=1}^{2}(y_{ij}-\bar{y}_i)^2 = \tfrac{1}{2}(y_{i1}-y_{i2})^2.)$$

•	•	•	•	•	N	•	N	•	N	N
•	N	•	•	N	N	N	•	N	•	•
N	N	N	N	•	N	•	N	N	N	•
N	N	•	N	N	N	N	N	N	•	•
N	N	N	N	•	N	•	•	N	N	•
•	N	N	•	N	N	N	•	•	N	N
N	N	N	N	•	N	•	•	•	•	•
N	•	•	N	•	N	N	N	N	N	N
N	N	•	N	•	•	N	N	•	N	•
•	N	N	N	N	N	N	N	•	•	N
N	N	N	•	•	•	N	N	•	•	N
•	•	N	•	•	N	•	N	N	N	N
N	N	•	N	•	N	•	•	N	•	N
N	•	N	N	•	N	•	•	•	•	•
•	•	•	•	•	•	•	•	•	N	•
•	•	•	•	N	•	•	•	N	N	N
•	•	•	•	•	•	•	•	•	•	N
•	•	N	•	N	•	•	•	•	N	•
N	•	•	N	•	•	•	•	•	•	•
•	•	•	N	•	•	•	•	N	•	•
•	•	•	•	•	N	•	•	N	•	•
•	•	•	•	•	N	•	N	N	•	N
•	•	•	•	N	•	•	•	•	•	N
•	•	N	•	•	•	•	•	•	•	N
•	•	•	•	•	•	•	N	•	N	N
•	•	•	•	•	•	•	•	N	N	N
•	•	•	•	•	•	•	•	•	N	N
N	•	•	•	•	•	•	•	•	N	•
•	•	•	•	•	•	•	•	•	N	•
•	•	•	•	•	•	•	•	•	•	•

FIG. 3

The value of Z is 0·131 which is obviously not significant. We conclude therefore that there is no evidence of lack of randomness brought out by our test. To complete the analysis we now rank the y's in order of magnitude and take the x's in pairs. This time we find $Z = -0{\cdot}198$ which again is not significant.

5. Freeman (1953) quotes an example given by Legg (1953) on the natural spread of nettlehead, a virus disease of hops. The 330 hop plants were set out in a lattice of 30×11. Fig. 3[1] shows the layout, the plants which had

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[1] The few gaps in the lattice have been ignored for purposes of illustration. They would not affect the calculations.

developed nettlehead by 1951 being marked N. We carry out an analysis similar to that of the preceding section on the N's, taking the distance of each affected plant from the middle of the lattice and using the upright lines of lattice as strips. With $k = 11$ and $N-k = 111$ and $Z = 0{\cdot}728$ we have a very significant result. A correction for the unequal groups can be made as described previously but this is hardly necessary for such a significant value of Z. We should have no hesitation from this analysis in saying that the plants which developed nettlehead are not randomly dispersed in the lattice.

6. We propose to develop the theoretical bases of this bivariate test elsewhere and at the same time discuss how the efficiency of the test will vary under differing circumstances. As has already been stated the test seems best when the number of plants per strip coincides with the number in a clump. Further investigations are being made to determine the ability of the test to detect non-randomness not only under various forms of clumping but also when there is some form of patchiness in the area under investigation even though the plants are distributed at random in the patches.

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Studies on the Physiology of Nodule Formation

V. Further Experiments on the Stimulating and Inhibitory Effects of Root Secretions

BY

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With eleven Figures in the Text

ABSTRACT

The influence of root secretions on nodulation of clover and lucerne in agar culture is examined using a technique of preplanting. Root secretions are shown to have two effects: nodulation of the seedling is induced at an earlier stage (stimulation), but takes place at a reduced rate (inhibition). These effects do not depend upon the presence of the bacteria in the preplanting phase of an experiment. The species used differed both in production of and reaction to secretions giving these effects. As a donating plant lucerne is more active than clover, and selected early nodulating lines of clover are more active than late lines. Stimulation of lucerne is promoted by lower levels of secretion than clover and early nodulating lines are less affected than late lines. The influence of secretions on nodulation rate is greater for lucerne than for clover at lower levels and does not differ between early and late nodulating lines. In contrast to lucerne, inhibition of nodulation on clover is proportional to duration of the preplanting suggesting that secretions accumulate during growth.

The hypothesis is put forward that the stimulating reaction represents an increase in the availability of the infective foci already present on the root and that the inhibiting reaction represents a reduction in the number of these foci.

INTRODUCTION

PRIMARY infection of the young seedling of clover generally takes place under favourable conditions at an early stage, with the first nodule forming on the main radicle near the zone of transition separating root and hypocotyl. On exceptional plants, however, primary infection may be much delayed with the first nodule appearing at a lower level on the main root or on a first-order lateral. These individual plant differences have been shown to be hereditary (Nutman, 1953).

In some species of host, earlier nodule formation on plants on which nodulation would be delayed may be brought about by growing a number of plants together (Thornton, 1929; Ludwig and Allison, 1935; Nutman, 1953). This stimulating effect on nodulation is transitory, fewer nodules finally being produced on the plants growing together than on plants growing singly. To explain these findings the hypothesis has been advanced that both effects are due to the accumulation of root secretions in the medium which are stimulating at a low concentration and become inhibitory at a higher concentration.

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In so far as inhibition of nodulation is concerned, data presented in Part IV of this series show that inhibitory effects were obtained by growing the interacting plants in succession as well as together in the same culture. In the present paper both these effects will be further investigated by this means. The technique of preplanting offers advantages over the growing of the interacting plants together in that the donating and reacting components are separate and competitive effects are largely eliminated.

MATERIALS AND METHODS

All plants were grown in $6 \times \frac{3}{4}$ in. test-tubes on agar slopes maintained at an approximate constant volume by periodic additions of dilute culture solution. The plant cultures were placed in wooden racks on the glasshouse bench and were shaded by slat blinds from direct sunlight. Further details of the technique are given in the earlier papers. The plant species used were: *Trifolium pratense* L., selected families of Montgomeryshire red clover; *Medicago sativa* L., Provence variety. Inoculation was made from agar cultures of *Rhizobium trifolii* Dang. strain ClF for red clover and *Rhizobium meliloti* Dang. strain AH_2 for lucerne; each strain being effective on its appropriate host.

Preplanting was carried out for varying periods and after the removal of the preplanted seedling with forceps the externally sterilized test seed was introduced on the following day. Aseptic technique was used throughout.

Two experiments were carried out on the effect of preplanting on nodule initiation and production.

RESULTS

EXPERIMENT 1

This experiment was designed to determine whether the presence of the bacteria during preplanting or subsequently had any influence on stimulation or inhibition.

Test sowings were made with lucerne and with early and late nodulating families of clover. A single preplanting period of 37 days was employed using lucerne and a single clover family. Inoculations of preplantings were made with either a clover or a lucerne strain of bacteria and in addition one set of controls was inoculated with both strains. A further set of controls was sown with paired plants.

The inoculation régime included treatments in which inoculation was delayed up to such time as nodulation was ordinarily complete on the test plants, viz. for 2, 5, 7, 10, 13, 16, and 20 days. Delayed inoculation has an effect on the number of nodules produced (Nutman, 1949) so that additional controls were set up covering the full inoculation schedule. The plan of the experiment is illustrated in the diagram (Fig. 1). Replication varied due to irregularities in germination and occasional fungal contamination. All combinations of plant type and treatment were employed giving a total of 550 individual test-plant cultures.

The age at which visibly distinguishable nodules first appeared on each seedling was noted and periodic counts of nodules and roots were made.

Results are set out in Tables I and II. The salient features which emerge are that the different inoculation treatment effects are quite negligible, whereas those due to preplanting and to family differences in clover are very highly significant. The remaining experimental results, viz. the trends associated with delay in inoculation, with and without preplanting are represented graphically in the figures.

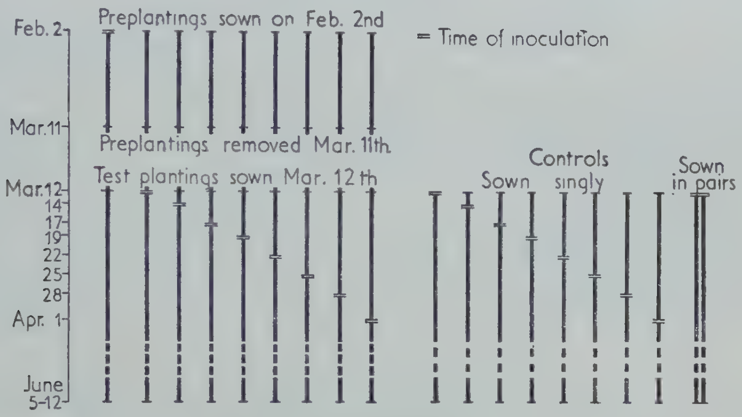

FIG. 1. Plan of Experiment 1.

Nodule stimulation

The statistical analyses were complicated by varying replication and heterogeneous variances.[1]

The analysis in Table I, given in terms of mean plant age at primary infection, is divided into three components (1) control treatments without preplanting, (2) control versus preplanted treatments, (3) between preplanted treatments.

For lucerne, preplanting with lucerne or clover advances the time of nodulation by about 6 days. This average amount of stimulation cannot be examined in the analysis of variance because of a significant preplanting species *v.* inoculation schedule interaction. However, there is no doubt as to its significance as will appear when the larger amount of material dealing with the effect of inoculation delay is considered. Stimulation occurs with both preplanting species and does not depend upon preinoculation.

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[1] For nodulation time there was a satisfactory linear relation for replicate variance against treatment mean except with the lowest four means for which the actual variances were used. The estimated variances for the other means were determined from the line estimate of replicate variance. An approximate analysis of the non-orthogonal data was made by giving each mean a weight inversely proportional to its variance ignoring errors in weights. Replicates of treatments shown not to be significantly different at any stage were pooled to give a new estimate of replicate variance which was contrasted with the corresponding reading from the line at the mean of the pooled data. The agreement was always satisfactory and the reading from the line was used for subsequent analyses.

The more comprehensive data for clover shows that the clover families differ among themselves in nodulation time, but these differences are not affected by the presence of a second strain of bacteria or by the presence of a second plant in a culture. The absence of any differences between control sets facilitates comparison between preplanted treatments in some of which double inoculation occurred.

TABLE I

Comparison of Times of Initiation of Nodules on Test Plants Inoculated at or Before Sowing

A. *Table of Mean Nodulation Times on Test Lucerne or Clover (in days from sowing)*

		Clover families			
	Lucerne	A	B	C	D
Controls					
1. Single test plants					
(*a*) single inoculum (strain ClF or strain AH_2)	23·2	14·0	16·3	20·8	16·4
(*b*) double inoculum (strain ClF and strain AH_2)	—	14·7	13·4	16·6	22·0
2. Paired test plants	23·7	13·9	15·9	17·1	19·6
Preplanted treatments					
1. Preplanted with lucerne					
(*a*) inoculated preplanting	20·7	11·8	12·4	13·5	16·8
(*b*) uninoculated preplanting	16·5	12·0	11·3	12·3	12·7
2. Preplanted with clover					
(*a*) inoculated preplanting	15·1	11·5	11·2	14·0	13·5
(*b*) uninoculated preplanting	17·5	11·6	14·5	11·0	16·5

B. *Analysis of Variance*

	Lucerne				Clover			
Source	d.f.	mean sq.	F	Signif. (*P*)	d.f.	mean sq.	F	Signif. (*P*)
1. *Control treatments*								
Host families	—	—	—	—	3	8·154	8·15	< 0·001
Treatments	1	0·901	< 1	n.s.	2	0·300	< 1	n.s.
Interaction	—	—	—	—	6	1·181	1·18	n.s.
Error	27	85·635			141	1·000		
2. *Control versus pretreatment*								
Host families	—	—	—	—	3	10·613	10·61	< 0·001
Preplanting	—	—	—	—	1	50·901	50·90	< 0·001
Interaction	—	—	—	—	3	1·394	1·39	n.s.
Error	—	—	—	—	190	1·000		
3. *Between pretreatments*								
(*a*) Host families	—	—	—	—	3	2·448	2·45	n.s.
Treatments	3	51·358	4·09	< 0·01	3	1·313	1·31	n.s.
Interaction	—	—	—	—	9	1·049	1·05	
Error	25	12·536			41	1·000		
(*b*) Preplanting species	1	33·267	2·65	n.s.	—	—	—	—
Inoculation	1	4·089	< 1		—	—	—	—
Interaction	1	56·343	5·48	<0·05	—	—	—	—
Error	25	12·536						

The effect of preplanting in stimulating nodule formation in clover is very highly significant (second analysis, control *v.* pretreatment) and varies with clover family. There is a suggestion that the smallest difference is associated with the smallest mean and vice versa; viz. that early nodulating families are less stimulated by preplanting than late families, but this effect does not reach statistical significance.

The third analysis (between pretreatments) shows that neither the species used for preplanting nor the presence of bacteria during the preplanting phase has any effect on the degree of stimulation obtained. It is also seen from this analysis that the marked family effects which appeared without pretreatment are now absent; so that stimulation is relatively greater for late than for early lines.

The remaining treatments concern delayed inoculation. Their object was twofold: to examine quantitatively any effect of the bacteria on the stimulating activity of the hypothetical secretion and to investigate directly the time interval which elapses between inoculation and visible nodule formation after varying periods of delay, in the presence and in the absence of root secretion.

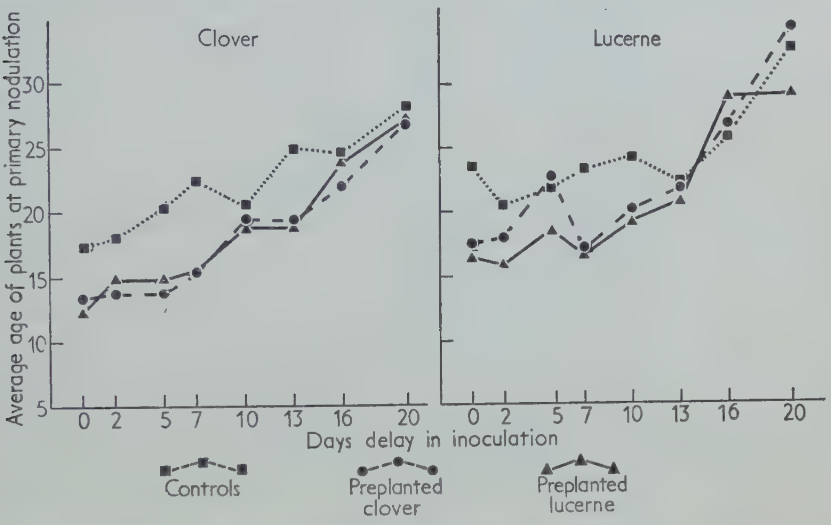


FIG. 2. Effect of delayed inoculation upon nodule initiation on clover and lucerne with or without preplanting.

The fact that time to nodulation was independent of presence or absence of bacteria during preplanting disposes of the first relation. The data, however, yield interesting information on the time taken for the initial stages of infection to be completed. The results are set out in Fig. 2 which shows the mean nodulation times for control and preplanted sets, with inoculation at sowing or after varying periods of delay.

On control sets inoculated at sowing, nodule formation on clover begins at about 17 days and on lucerne at about 22 days and is further delayed by postponing inoculation, until with 20 days delay nodulation does not take place until about the 28th day for clover or the 33rd day for lucerne.

With preplanting, time of nodulation is advanced throughout, but to a decreasing extent with increasing periods of delay in inoculation until at 20 days it is not significantly ahead of controls. This result might be anticipated since at between 16 and 20 days the control sets of both species may in any case be open to infection because of sufficient secretion by the test plants themselves.

The first stage of nodule formation

The time interval between inoculation and nodule formation is in some respects a more fundamental criterion than age of plant and it is of interest to determine its minimum value.

With this in view the nodulation data for clover and lucerne, with and without preplanting, are represented in Fig. 3 in terms of this interval (which will be referred to as presentation time). In addition to the treatment means the actual minimum values found among the replicates for each species of test plant are shown on the figure. The letters P and/or C beside each point indicate that the minimum concerned is from a preplanted treatment or control respectively.

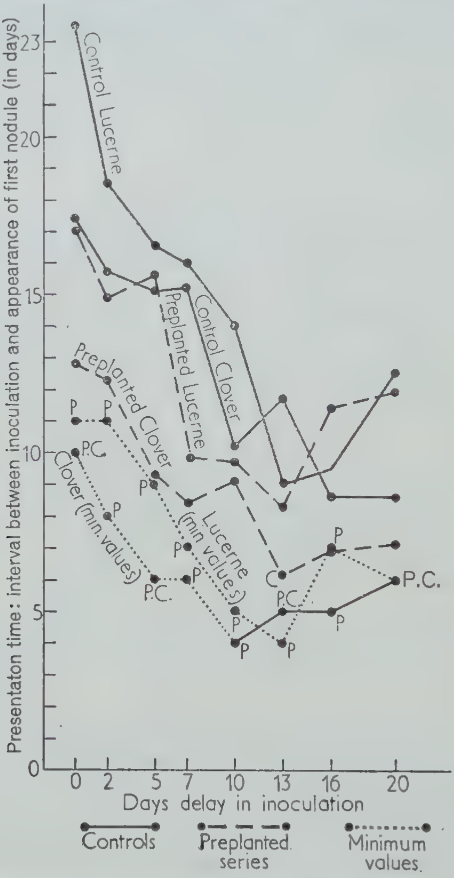


FIG. 3. Effect of delayed inoculation with or without preplanting upon the interval between inoculation and the appearance of nodules (presentation time).

Three interesting relations are evident:

1. For both species the average presentation time tends to reach a minimum value with a delay in inoculation of between 10 and 13 days independently of whether or not preplanting was employed.

2. The effect of preplanting is to reduce this minimum period by 2 or 3 days for both species.

3. The lowest curves show (i) that the minimal presentation time for both species is 4 days; and (ii) that some of these minimal values are given by control plants; thus, in spite of the fact that preplanting reduces the average presentation time, individual plant differences may transcend this effect.

These results emphasize that the requirements for nodulation in clover and lucerne are very similar. A minimum period of about 4 days must elapse for either species to become visibly nodulated from a suspension of bacteria added to the root system. This minimum (which may be compared with a minimum period of 5 days for lucerne found by Thornton, 1929) is attained, however, only if the inoculation is made at between 10 and 13 days from germination and is more frequently found if the stimulating root secretions are also present. Clover and lucerne would appear to differ only in the frequency with which infection occurs at this minimum period.

Infection may therefore be regarded as an event which may not take place even under optimal conditions. This is in conformity with the general hypothesis of these studies, viz. that infection is limited to discrete foci on the root which may or may not be available for infection at a given time. The action of the stimulating substance might be to induce the appearance of these foci or to be concerned in other stages of the infection process; e.g. to provide material for bacterial development outside the plant. It seems probable that four days are required to elapse under the most favourable conditions between invasion by the bacteria and the development of a nodule of visible size, suggesting that invasion takes place immediately upon exposure of a site.

Inhibition of nodulation

The data were analysed as the logarithm of the number of nodules (+1) found at the end of the experiment, this transformation being successful in making variances homogeneous in all treatments. The results for all treatments inoculated at or before sowing of test plants are shown in Table II.

For both kinds of test plant inhibition by pairing (analysis 1*b*) or by preplanting (analysis 2) is confirmed, but there is no effect of preinoculation (analysis 3) or of the presence of both strains of bacteria (analysis 1*a*).

The influence of delayed inoculation may be followed in Fig. 4 for lucerne and clover.

Detailed consideration of trends is not possible because of scatter but it would appear that the increase in nodule number following delayed inoculation at 13, 16, and 20 days occurs independently of inhibition. This increase was noted in previous work (Nutman, 1949) and has been ascribed to an increase in the rate of appearance of infective foci by the formation, in delayed sets, of lateral roots which now give rise to further foci. Previously it was found for clover that a delay of about 20 days produced maximum stimulation, longer delay leading to the production of fewer nodules. In lucerne the maximum stimulation is greater than in clover and occurs earlier on both the control and preplanted sets.

TABLE II

Comparison of Numbers of Nodules Produced on Test Plants Inoculated at or Before Sowing

A. *Table of Means (Harmonic)*

	Lucerne	Clover families			
		A	B	C	D
Controls					
1. Single test plants					
(*a*) single inoculum (strain ClF or strain AH_2	9·9	37·7	30·4	22·3	24·7
(*b*) double inoculum (strain ClF and strain AH_2)	—	25·5	37·1	23·2	19·6
2. Paired test plants	5·2	14·2	12·1	17·2	13·9
Preplanted treatments					
1. Preplanted with lucerne					
(*a*) inoculated preplanting	7·0	22·8	16·8	14·9	19·3
(*b*) uninoculated preplanting	5·8	19·5	12·6	21·1	18·9
2. Preplanted with clover					
(*a*) inoculated preplanting	7·9	24·9	22·8	18·4	32·8
(*b*) uninoculated preplanting	6·7	17·8	30·3	28·9	16·0

B. *Analysis of Variance* (*on* log N + 1)

	Lucerne				Clover			
Source	d.f.	Mean sq.	F	Signif. (*P*)	d.f.	Mean sq.	F	Signif. (*P*)
1. *Within control treatments*								
(*a*) Host Families	—	—	—	—	3	0·151	3·23	< 0·05
Treatment (single *v.* double inoculum)	—	—	—	—	1	0·032	< 1	n.s.
Interaction	—	—	—	—	3	0·061	1·30	n.s.
Error	—	—			70	0·047		
(*b*) Host Families					3	0·041	< 1	n.s.
Treatment (single *v.* paired plants)	1	0·250	3·76	0·06	1	1·618	37·90	< 0·001
Interaction	—	—	—	—	3	0·179	4·19	< 0·01
Error	50	0·066			140	0·043		
2. *Control* (*single plants* v. *pretreatment*)								
Host Families	—	—	—	—	3	0·087	1·68	n.s.
Treatment	1	0·079	1·20	n.s.	1	0·440	< 1	n.s.
Interaction	—	—			3	0·078	1·51	n.s.
Error	50	0·066			110	0·052		
3. *Between pretreatments*								
Host Families	—	—	—	—	3	0·016	< 1	n.s.
Treatment	3	0·017	< 1	n.s.	3	0·053	< 1	n.s.
Interaction	—	—			9	0·040	< 1	n.s.
Error	50	0·066			40	0·060		

The results show that nodule stimulation due to delayed inoculation and nodule inhibition due to preplanting are independent. This is in conformity with the hypothesis; the root secretions having a direct effect on the development of foci and the inoculation treatments affecting the partitioning of these foci between nodules and lateral roots.

Other than an indication of clover family differences in susceptibility to the stimulatory secretion (Table I, analyses 2 and 3), this experiment gives little information on host-plant differences, either as donating or reacting plants.

This is particularly the case in regard to the lucerne treatments which were poorly replicated owing to irregularities in germination.

This aspect is examined in the following experiment.

EXPERIMENT 2

This experiment was designed to investigate duration of preplanting time using single preplantings lasting for 5, 10, 20, 40, and 80 days and 1, 2, and 4 successive replantings each lasting for 10 days. Early and late lines of clover and Provence lucerne were employed in both preplanting and in testing the effects of preplanting, using all combinations of treatment and plant type (giving a total of 450 plant cultures in test sowings). Each sowing (preplanting and test) was inoculated with large inocula of bacteria, adjusted turbidometrically to a standard density. Extra controls were again set up to test the effect of the presence of both strains on the nodulation of each species; as in experiment 1, preinoculation with either strain was without effect. Sowings were arranged so that all test material was sown on the same day.

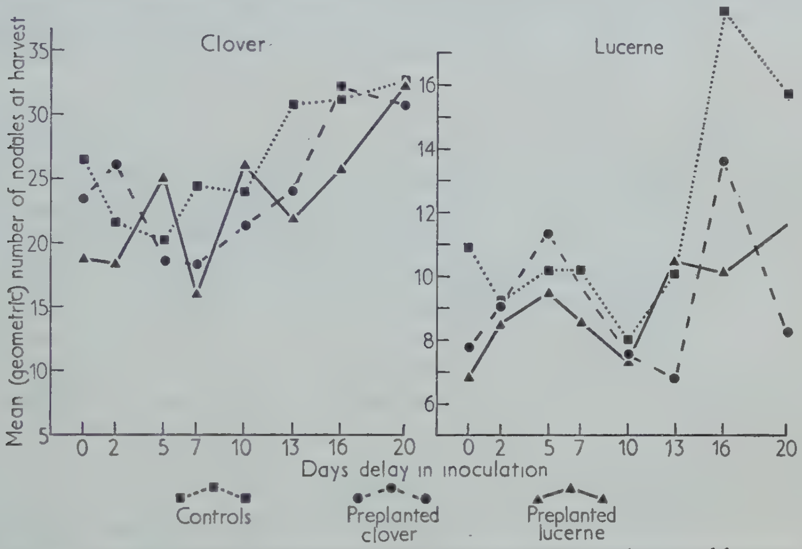


FIG. 4. Effect of delayed inoculation upon nodule density on clover and lucerne.

Nodule stimulation on lucerne

The effects of the various preplanting treatments in experiment 2 using lucerne as a test plant are shown in Fig. 5 in terms of seedling age (average) at the time the first nodule appeared; this will be referred to as nodulation time or age. The abscissa gives on a geometric scale either the duration of the periods of preplanting or the number of replantings employed. Separate curves are drawn for the sets preplanted with lucerne or with early or late nodulating lines of clover. A horizontal line indicates primary nodulating age of control seedlings (with no preplanting).

The results show, in confirmation of experiment 1, that preplanting with lucerne, early clover, or late clover shortens the period of the seedlings' freedom from infection. This stimulatory effect is given by as little as 5 days preplanting. There is an indication that longer periods of preplanting are increasingly stimulatory, but this trend is not statistically significant. Increasing the number of replantings shows no consistent cumulative effect. Except for irregularities due to irregular replication, the relative stimulating effects on lucerne clearly differ for the two species, lucerne showing most stimulation and late nodulating clover least. The larger stimulatory effect of lucerne compared with clover was also remarked in the previous paper (Table VIII, p. 114) although it did not there reach a level of statistical significance.

Nodule stimulation on clover

The results shown in Fig. 6 for both early and late lines of clover combined show interesting differences in comparison with lucerne, and indicate that clover is somewhat less susceptible to low concentrations of secretion. With only 5 days of preplanting the stimulatory effect is slight and only becomes fully developed by a preplanting period of 20 days. These results suggest that the stimulating substance is rapidly produced by the developing seedling and reaches an optimum concentration after 10–20 days' growth. The curves on the right side of Fig. 6 show that successive replantings, each of 10 days' duration, do not augment the stimulating effect. This may also be because further stimulation does not take place above a certain concentration of root secretion in the medium.

The three kinds of plant material used for preplanting showed no differences in stimulating nodulation on clover: they presumably all produce sufficient of the hypothetical stimulant at an early stage to give maximal effect. This result is again in contrast to lucerne.

In Fig. 7 the stimulating effects of preplanting species and strains may be compared on early and late nodulating lines separately, and in confirmation of the results found in experiment 1 (Table I) marked family differences were observed. The control plants of these lines differed by 3½ days in average age at primary infection. The maximum stimulating effect on late lines was about 6½ days and on early lines about 3½ days, so that after preplanting early and late lines could no longer be distinguished. This obliteration of family differences was more noticeable after longer periods of a single preplanting than after a number of successive replantings of short duration. Late lines therefore differ from the early ones only in their response to root secretions; viz. in being relatively more affected by a given concentration of stimulant and in producing it less rapidly. In this connexion it is of interest that early nodulating lines are known to be more active than late lines also in the production of the inhibitory secretion (Nutman, 1953, p. 109).

In this experiment the early lines were also subject to stimulation, presumably because the selection used did not represent a fully early line, i.e. one in which each seedling would be able to produce sufficient secretion to nodulate at the earliest possible stage.

Nodule inhibition on lucerne

The influence of the various preplanting treatments on the number of nodules developed on lucerne at the end of the experiment are shown in Fig. 8. The amount of inhibition found is largely independent of treatment. Neither increase in time, beyond a period of about 10 days, nor in number of replantings has any progressive effect on inhibition. Also, the three kinds of preplanting material do not differ in their influence. If the results for lucerne are to be explained in terms of activity of a root secretion provided by the donating plants, it would appear that this substance acts at a low concentration since the quantity secreted in 10 days is sufficient for maximal inhibition (number of nodules, Fig. 8) as well as for maximal stimulation (mean age at nodulation, Fig. 5).

The nodules of lucerne are occasionally branched (equivalent in this experiment to the production of three extra meristems per plant). Branching was distributed at random in the experiment so that the same relations were found to hold if the number of nodule meristems were considered instead of the number of root infections leading to nodule development.

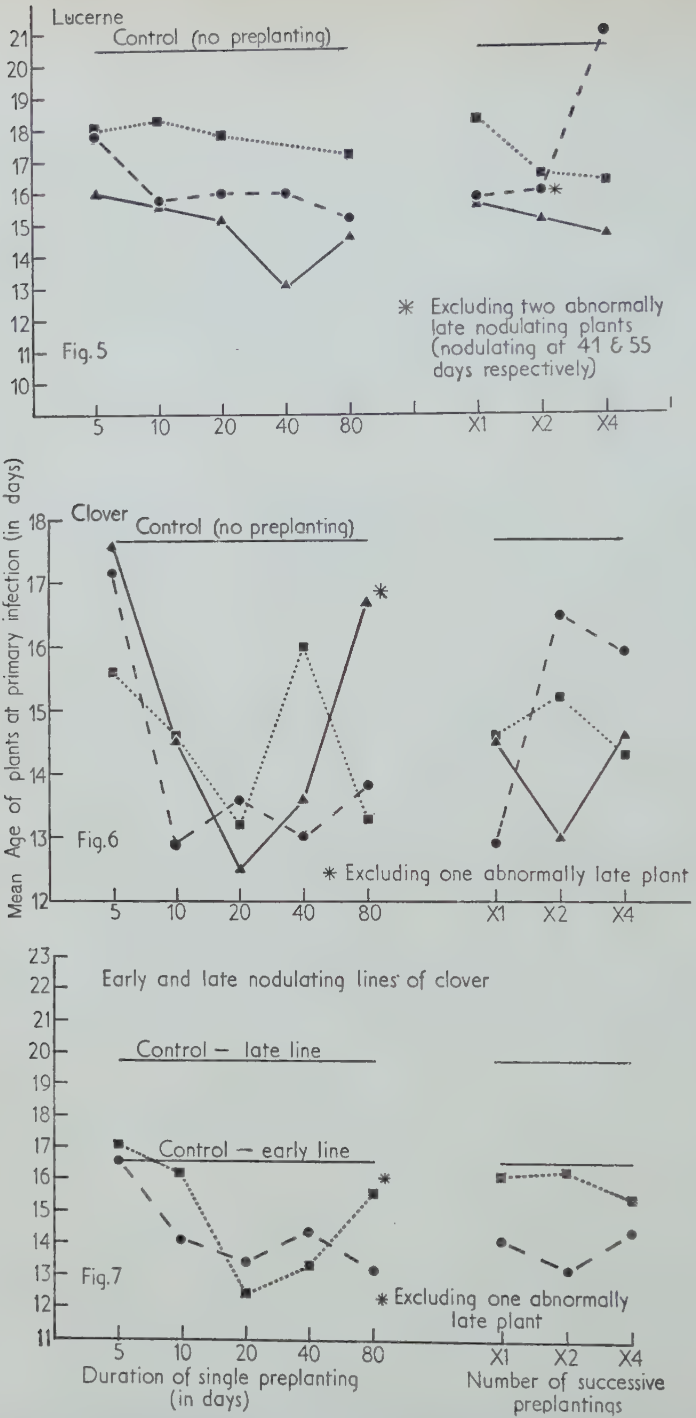
Nodule inhibition on clover

The results for inhibition on clover are given in Figs. 9 and 10.

The degree of nodule inhibition increases continuously with the duration of preplanting and in this respect differs markedly from lucerne. The inhibitory effect on nodule number of clover of varying pretreatment times also differs radically from the stimulating effect in terms of the age of plants at inoculation (Figs. 6 and 7). Inhibition is hardly evident with 10 days preplanting, a treatment which gives nearly maximum acceleration of nodulation on the young seedling (Fig. 6). With 80 days preplanting nodulation is reduced to half and corresponds to that found on plants grown together instead of successively. A comparison of 1, 2, and 4 replantings on clover (early and late lines combined, Fig. 9) using different preplanting species gives concordant results in showing increasing inhibition with increase in the number of replantings.

In contrast to inhibition on lucerne the three kinds of preplanting material appear to differ; lucerne is somewhat more inhibitory than the early and late lines of clover, the early nodulating lines being slightly more inhibitory on the average than the late nodulating lines.

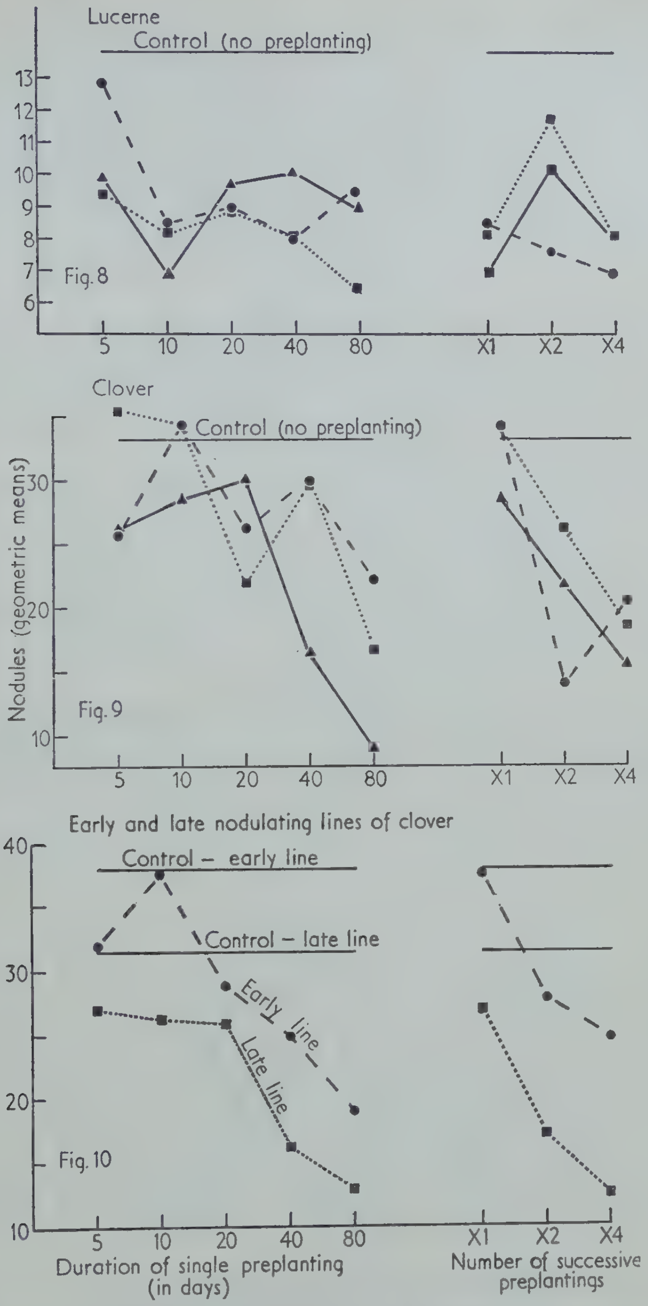
A comparison of inhibition by all species on early and late lines of clover is given in Fig. 10. The progressive effect of the pretreatments is shown by the slopes of the lines and is clearly the same for each type of plant material. The early nodulating families were here more abundantly nodulating than the late lines. This relation was, however, fortuitous; other early and late families might have been chosen showing opposite effects (see Part IV). That early and late nodulating lines are equally susceptible to the action of these root secretions was also concluded from earlier experiments (Nutman, 1953, p. 108).



Key to preplanting species in Figures 5 and 6: ▲ Lucerne, ● Early nodulating line of clover, ■ Late nodulating line of clover. In Figure 7 the symbols refer to test lines.

FIGS. 5–7.

The stimulatory effect of preplanting upon nodule initiation on lucerne (Fig. 5) and on clover (early and late lines combined, Fig. 6; early and late lines separately, Fig. 7).



Key to preplanting species in Figures 8 and 9. ▲ Lucerne, ● Early nodulating line of clover, ■ Late nodulating line of clover. In Figure 10 the symbols refer to test lines.

FIGS. 8–10.

The inhibitory effect of preplanting on the numbers of nodules formed on lucerne (Fig. 8) and clover (early and late lines combined, Fig. 9; early and late lines separately, Fig. 10).

These results may be explained on the hypothesis that an active agent secreted by the root is stimulating for nodule inception at low concentration but is inhibitory at a higher level. The active secretion is produced more readily by lucerne than by clover and less readily by late nodulating lines of clover than by early lines. This order of activity was also found in earlier experiments on the mutual inhibition of plants grown in association as well as in the stimulatory effects of root secretions on the initiation of nodulation on lucerne (Fig. 4).

DISCUSSION

The above experimental results show that the stimulatory and inhibitory effects previously ascribed to the mutual interaction of plants when growing together can be equally well demonstrated in the absence of the secreting host plant. Clover and lucerne differ in production of, and reactivity to, the root secretions responsible for these effects.

The results suggest that the production of secretion is continuous during growth and takes place rather more rapidly from lucerne than from clover roots, and less rapidly from late nodulating lines than from early lines. Differences also appear in plant susceptibility, lucerne being more readily stimulated than clover. These relations may explain the earlier finding that stimulation will sometimes occur in cultures of lucerne containing two plants but not in paired clover cultures (Part IV).

On the other hand the number of nodules produced on lucerne is less affected by level of inhibitor than is clover, even in the early stages of seedling growth. Quantitative relations of these kinds may explain the different degrees of inhibition found when growing various species of plant together (Part IV, p. 117). There is nothing in the data to suggest any degree of specificity either in secretion or response to secretion, although the consistent preplanting species differences in Fig. 5, which are independent of time, suggest that qualitative as well as quantitative relations may be of importance. There is no evidence that the bacteria play other than an unspecific role in these reactions.

Of host differences the most significant is that between early and late nodulating lines of red clover. All results point to the conclusion that late nodulating lines are less active in secretion, whether this is determined by their stimulating or inhibiting effects. These results are in accord with the simple hypothesis that stimulation and inhibition are influenced by different amounts of the same substance or group of substances, but do not exclude the alternative hypothesis that more than one substance may be involved. Also it is clear that high as well as low concentrations of secretion will be stimulatory.

Relation between stimulating and inhibiting effects

Plants which are subjected to conditions which induce earlier nodulation than on control plants have more nodules at an early seedling stage. This is illustrated in Fig. 11 which gives the progress of nodule formation on young plants of clover and lucerne in the principal treatments in experiment 2. The apparent stimulation of nodule number by pretreatment on plants younger than about 20 days depends wholly upon earlier nodule initiation.

Comparison of the stimulating and inhibiting reactions raises the question as to whether inhibition is a reaction to the external secretion or an internal reaction of the plant consequent upon stimulation. It may, for example, be argued that the nodules initiated early in the presence of root secretions inhibit subsequent nodulation without the further intervention of root secretion. The data in Tables I and II are relevant to this question. They show that plants grown in pairs are mutually strongly inhibitory but are not stimulatory for the earlier initiation of nodules. The inhibitory effects under these conditions have been shown to be unaffected by competition for nutrients, &c. (see Part IV). These results suggest that stimulation and inhibition are independent reactions of the host to root secretions. This conclusion is also supported by Fig. 11 which shows that pretreatments giving maximum stimulation on clover are least inhibitory and vice versa.

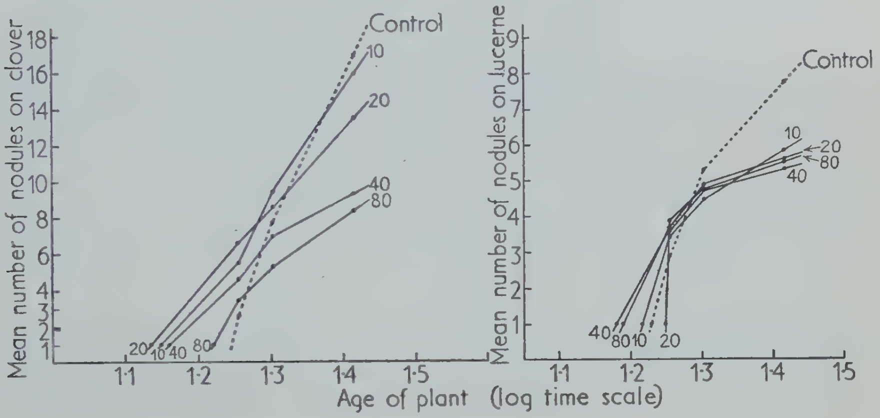


FIG. 11. Progress of nodulation on clover and on lucerne with and without preplanting.

The results are not at variance with the view that infection takes place at discrete points or foci on the root, with stimulatory amounts of secretion increasing in some way the availability of these foci rather than their number, and inhibitory amounts affecting their abundance. This is suggested by a tendency, which is more marked for lucerne, for lateral root emergence to be inhibited rather than stimulated by preplanting, even in the young seedling as shown by data given in Table III. The suppression of lateral rooting at an early stage has been noted as a feature of inhibition by associated growth and indicates that inhibition operates from the outset but cannot otherwise be observed because of the increased availability of the foci which is characteristic of the stimulating reaction. In confirmation of results found previously (Nutman, 1948) lateral roots are more numerous on young clover seedlings (16 days old) not yet inoculated than on those already forming nodules. This suggests that the first formed nodules arise at points which would otherwise give rise to lateral roots. On lucerne this difference does not appear presumably because on this host nodulation has hardly begun at 16 days.

A further result of interest is that the *minimum* time (4 days) after inoculation for the macroscopic results of infection to show is the same for clover and lucerne in spite of wide and in some respects contrasted differences in nodulation. A closer examination of the early infection processes should help in elucidating the mechanism of the stimulating and inhibitory reactions and in confirming or refuting the above analysis.

TABLE III

Number of Lateral Roots on Test Seedlings of Lucerne and Clover at 16 days (Harmonic means)

Treatment	Inoculated at or before test sowing (significance of differences from control indicated)	Uninoculated, viz. 16 and 20 day delay treatments (significance of difference from control indicated)	Significance of differences due to inoculation
	Lucerne		
Control . . .	11·59	9·39	
Preplanted lucerne .	6·63***	7·37 n.s.	n.s.
Preplanted clover .	6·87**	4·58***	***
	Clover		
Control . . .	5·85	6·66	
Preplanted lucerne .	4·04*	9·12*	***
Preplanted clover .	3·94 n.s.	7·55 n.s.	**

* $P < 0·05$ ** $P < 0·01$ *** $P < 0·001$

Analysis of Variance

Source	d.f.	Mean sq.	F.	Signif. (*P*)
		Lucerne		
Treatments . .	2	0·1352	11·79	$P < 0·001$
Inoculation . .	1	0·0457	3·98	$P < 0·05$
Treat. by Inoc. .	2	0·0823	7·18	$P < 0·01$
Error. . .	49	0·0115		
		Clover		
Treatments . .	2	0·0378	< 1	n.s.
Inoculation . .	1	1·2028	17·17	$P < 0·001$
Treat. by Inoc. .	2	0·2593	3·70	$P < 0·05$
Error. . .	159	0·0701		

SUMMARY

Nodulation on lucerne and on clover was examined in fresh agar cultures and in cultures from which previously grown seedlings had been removed. The following relationships were found:

1. For both species preplanting stimulates the earlier formation of nodules.

2. Maximum stimulation of earlier nodulation on lucerne and clover was associated with preplanting for 5 days or 20 days respectively. Longer periods of preplanting are without further effect on lucerne and tend to reduce stimulation on clover.

3. Selected late nodulating lines of clover are more subject to stimulation than early nodulating lines. With preplanting these host-strain differences disappear.

4. For both species the stimulating effects of preplanting are transitory, giving place to an inhibition of the number of nodules produced. For lucerne the degree of this later inhibition was independent of preplanting time beyond a period of 5 days, but for all clover lines inhibition was increased by longer preplanting.

5. Lucerne was more active as a donating species than was clover and early nodulating lines of clover more active than late lines.

6. The stimulatory and inhibitory effects of preplanting were unaffected by the presence of nodule bacteria of either cross-inoculation group in the preplanted culture.

7. An examination of the influence of delayed inoculation on nodule formation on control and preplanted seedlings showed that clover and lucerne attain a state of maximum liability to infection at about two weeks. Nodule formation at this time occurs within a minimum time of four days for both species.

8. These findings are discussed in relation to the hypothesis previously advanced that infection on the root is restricted to certain meristematic foci in the root. It is suggested that root secretions produced from the preplanted species affect both the number of foci and their availability for infection.

ACKNOWLEDGEMENTS

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Leaf-area Growth in *Pelargonium zonale*

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With one Figure in the Text

ABSTRACT

The curve of growth in area of *Pelargonium* leaves against time is strongly asymmetrical, and cannot be fitted by the usual logistic curve. It is shown that the Gompertz curve provides an excellent fit, and the significance of its parameters is discussed.

INTRODUCTION

IN comparing the growth of leaves under different conditions, it is convenient to be able to fit the growth curves by some mathematical expression, however arbitrary, defined by a small number of parameters; Gregory (1921) showed, for example, that the logistic (autocatalytic, Verhulst-Pearl) curve serves very well for *Cucumis* leaves. The leaves of *Pelargonium*, however, reach their maximum growth-rate after about one week, continue growing for some 8 weeks, and under normal conditions survive in all for about 16 weeks. The rate curve is thus strongly asymmetrical, and cannot be even approximately fitted by the logistic curve. Generalized 'skew logistic' curves have been devised, but are difficult to fit; and the purpose of this brief note is to report the excellent fit obtained in this case with the Gompertz curve.

The equation of the curve is normally given as $y = Ka^{b^t}$; it is easily fitted (Croxton and Cowden, 1947) in the form

$$\log_{10} y = \log_{10} K + b^t \log_{10} a.$$

K, a, and b being thus determined, the theoretical rate curve can be drawn from the expression:

$$\frac{dy}{dt} = (\ln a)\,(\ln b) y \,.\, b^t$$

with a maximum rate of $-\frac{K}{e} \ln b$, when y (leaf size) $= \frac{K}{e}$ at a time given by

$(b^t \ln a = -1)$.

For use in comparisons, some interpretation of the parameters is desirable. The curve is asymptotic upwards to ($y = K$) so long as both a and b are $+ve$ and < 1 (which will always be the case for curves of leaf-growth) and K thus represents the final size attained. It tells us nothing that could not more easily have been obtained from the original data.

Now, let the time of the first observation be taken as zero. Then, if

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$a < 1/e$ ($\equiv 0{\cdot}3679$), the maximum rate will occur after the beginning of the observations, and the rate curve will first rise, then fall; if $a \geqslant 1/e$ the maximum will occur at or (theoretically) before the beginning of the observations, and the rate curve will fall from the start. This information, too, could be obtained from the original data with little trouble.

There remains b. If the equation is rewritten in the form

$$\frac{dy}{dt} = -(\ln b)[y(\ln K - \ln y)]$$

it will be seen that b represents the degree of dependence of the rate of growth at any moment upon both the size at that moment and the extent to which this falls short of the maximum size which will be attained if conditions remain unchanged. This may well be of physiological interest, and we have investigated the behaviour of the parameter b when leaves of the same species are grown under conditions sufficiently diverse to give widely different values of K.

EXPERIMENTAL RESULTS

Potted plants of *Pelargonium zonale* var. 'Paul Crampel' were grown under three different watering régimes: (1) normal daily watering (leaves A and B); (2) normal watering until cuttings were satisfactorily established (carrying 8 to 10 leaves), then no further watering (leaves C and D); (3) as (2), but after 5 weeks' drying, normal daily watering resumed (leaves E and F). When water is withheld from potted *Pelargonium* plants, all mature leaves are quickly shed; but each apex produces one or two small, tough, dark-green leaves which may persist for as long as 26 weeks. C and D are leaves of this type; and such leaves retain for a long time the capability of expanding rapidly when watering is resumed, as in the case of leaves E and F. The observations are incidental to a wider investigation into the physiological effects of drying (Amer, 1955) which will be published in due course. For the purpose of this note we have presented only weekly readings of leaf area.

TABLE I

Parameters of Gompertz Curves Fitted to Growth in Area of Pelargonium Leaves under Different Watering Régimes

Leaf	Régime	K	a	b
A	Normal	78·86	0·0617	0·4021
B	Normal	80·74	0·0633	0·4217
C	Dry	4·60	0·4019	0·5667
D	Dry	5·75	0·1352	0·4379
E	Dry, then normal			
	(i) dry	7·93	0·2668	0·5164
	(ii) normal	56·89	0·1725	0·4563
F	Dry, then normal			
	(i) dry	6·77	0·3832	0·4429
	(ii) normal	48·60	0·1804	0·4397

The actual results, the fitted curves, and the calculated rate-curves are given for all six leaves in Text-fig. 1. The values of the parameters K, a, and b for the fitted curves are summarized in Table I.

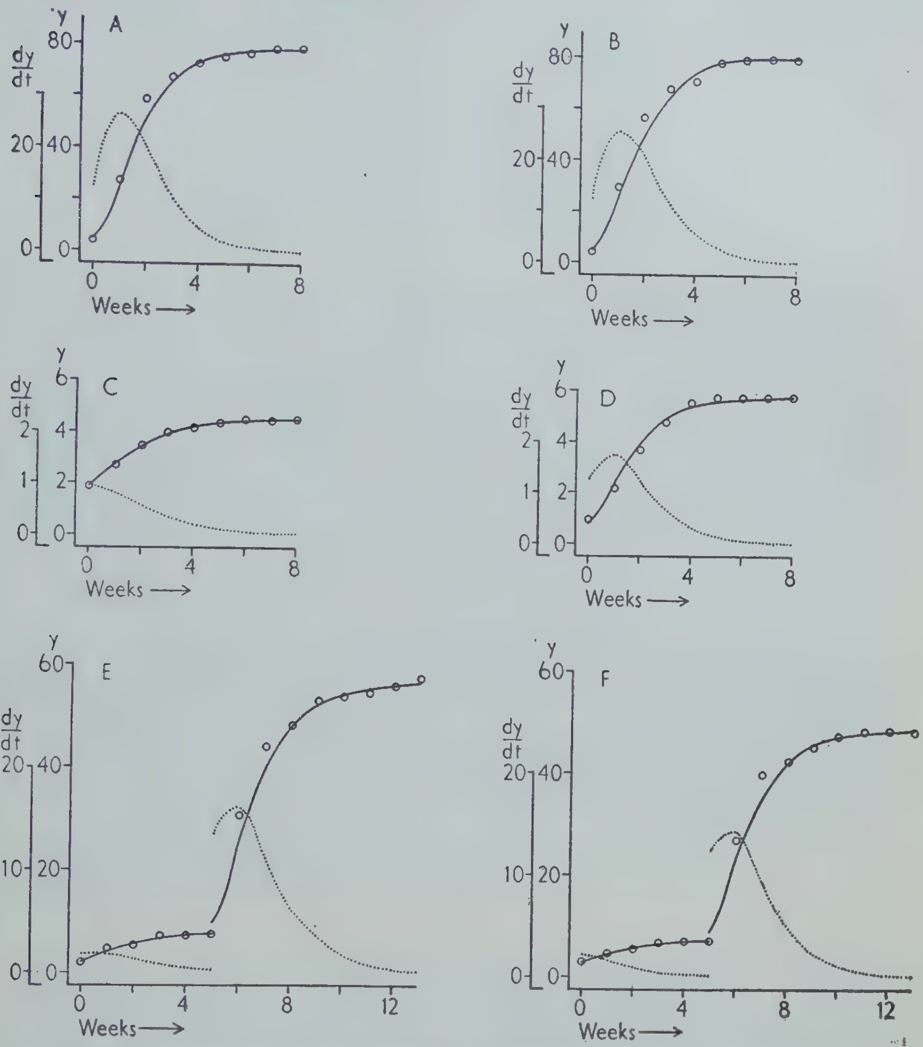

FIG. 1. Leaf-growth curves for *Pelargonium*. A, B, &c., represent different leaves (see Table I); circles represent *measured* areas; continuous lines represent the fitted Gompertz curves; dotted lines represent *calculated* rate curves. Ordinate scales: y in cm.^{2}, dy/dt in cm.^{2}/week.

DISCUSSION

The fit of these curves is obviously very satisfactory, and the usefulness of the Gompertz curve adequately confirmed. However, the most striking feature of the results is the constancy of the parameter b under widely different growth conditions; the four values for dry conditions seem to be slightly the higher, but this difference is not significant. In fact, b appears to be truly characteristic of the species. We had wondered whether any useful comparison could be made with the corresponding parameter of the logistic curve, but such comparison is complicated by the fact that b, as the equations are normally written, is not dimensionless. It can be made so by rewriting the two equations in the form:

$$\text{(logistic)}\ \frac{dy}{dt} = \frac{cb}{K}[y(K-y)]$$

$$\text{(Gompertz)}\ \frac{dy}{dt} = -c(\ln b)\left[y \ln \frac{K}{y}\right]$$

where c has dimensions $[T^{-1}]$.

If this transformation is applied to Gregory (1921) text-fig. 5[1] we find $b = 0{\cdot}460$. This happens precisely to equal the mean of the eight values of b given in our Table I; but these are weekly values, and the corresponding daily values for comparison with *Cucumis* will be given by the seventh root of each. The mean daily value of b then becomes 0·894. Data for other species would be needed before these figures could be usefully discussed further.

[1] There is a misprint in the legend of this figure: the numerator of the expression on the left-hand side of the equation should be x, not 236.

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Experimental and Analytical Studies of Pteridophytes

XXXVI. Further Experiments on the Developmental Potentialities of Leaf Primordia in *Dryopteris aristata* Druce

BY

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With Plates XII–XIV and forty Figures in the Text

ABSTRACT

On inactive apices of *Dryopteris aristata*, when young visible primordia which are not yet determined as leaves are isolated by four shallow incisions they undergo regression and disappear; but if the adaxial incision is subsequently deepened a proportion of the primordia are stimulated into a renewal of growth. About three-quarters of this number developed as buds, the rest as leaves, some of which were of radial symmetry; others became dorsiventral, but were sometimes abnormally orientated. Such primordia usually underwent a phase of radial symmetry during development. Subsequent deepening of the abaxial or radial shallow incisions was usually without effect.

Problems of leaf determination, symmetry, and orientation are examined in some detail. It is suggested that in specimens where the isolated primordia developed as leaves the experimental treatments have merely fortuitously reproduced the regulative effects normally exercised by the shoot apex and adjacent primordia. These may be specific relationships between the size and growth capacity of the primordium and that of the shoot apex on which it is formed.

Three phases in the development of a leaf primordium of this species can now be distinguished: inception as a growth centre, determination as a foliar organ of limited growth, and development as an organ of dorsiventral symmetry. Under normal growth conditions radial leaf development is never observed, but when growth is unusually slow it may become manifest. In other species the temporal relationships between these phases of development may differ so that they cannot be differentiated by experimental means.

INTRODUCTION

IN earlier experiments it was shown that in shoot apices of *Dryopteris aristata* Druce (*D. austriaca* (Jacq.) Woynar) which are not in active growth, isolation by four shallow incisions of young primordia not yet determined as leaves may cause them to cease growth and to disappear (Wardlaw and Cutter, 1954, 1956). The inception of prospective leaf primordia may also be prevented in this way, without injury to the sites of the growth centres. By contrast, isolation of comparable leaf primordia and of prospective leaf sites by similar but deep incisions, or by a single wide and deep adaxial incision, penetrating through the prestelar tissue into the pith, causes them to develop as buds (Wardlaw, 1949*b*; Cutter, 1954, 1956). It was therefore considered that isolation of such leaf primordia by four shallow incisions, followed by deepening of the adaxial cut at a later stage, might throw light on the poten-

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tialities for growth of the primordia, and, in particular, on the duration of such potentialities. On the basis of earlier results it might be expected that the deepening of the adaxial incision might provide a stimulus to a renewal of activity by any isolated primordia with residual growth potentialities. Furthermore, this effect might also conceivably be achieved by deepening the radial incisions instead, since Wardlaw (1949*b*) showed that isolation by deep radial incisions resulted in increased growth of a leaf primordium. The results of such experiments are described and discussed in some detail below.

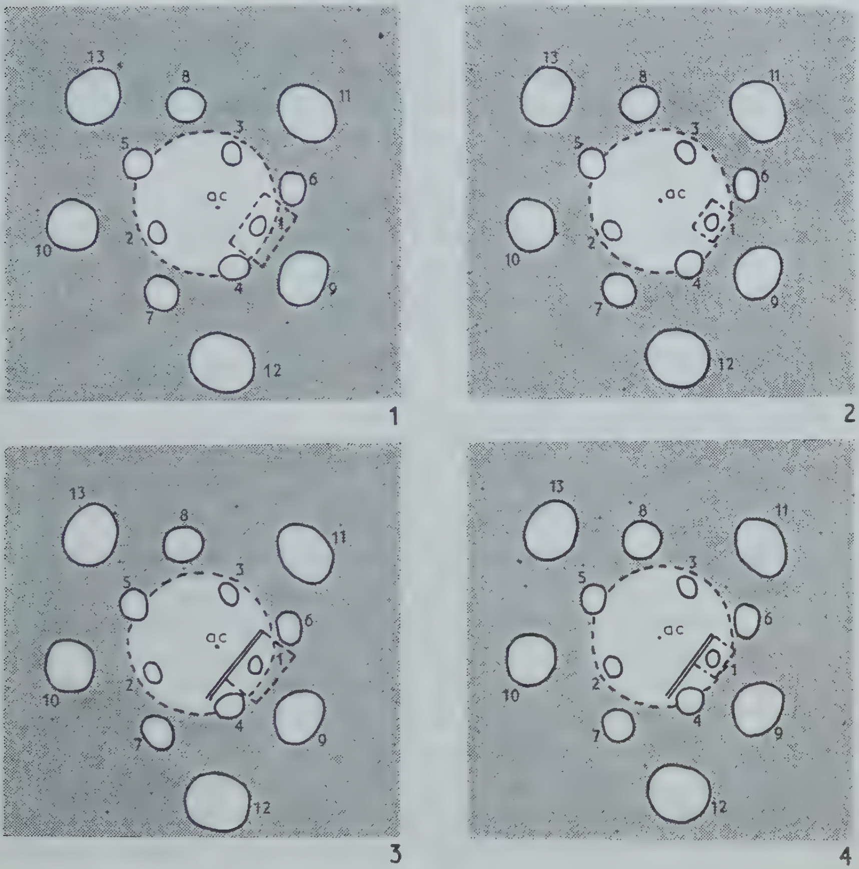
Materials and Methods

Large apices of *D. aristata* collected during the inactive season were laid bare as described in earlier papers (Wardlaw, 1944), and maintained in pans of damp peat in the laboratory. The pans were sometimes kept in a room maintained at 21°±1° C. during the winter.

Leaf primordia and presumptive leaf sites were isolated by four shallow incisions which, in general, penetrated only the superficial layer of cells of the apex, on either large or small plugs of the type indicated in Text-figs. 1 and 2. Occasional specimens in which the isolated primordia continued to grow and develop as leaves after this treatment were rejected; in the case of those in which the primordia ceased growth and disappeared the adaxial incision was deepened at a later date, usually when the primordium was considered to be just at the point of disappearance. The cut was deepened along the line of the original shallow incision, over a considerable width (Text-figs. 3 and 4); in the case of primordia isolated on small plugs, the adaxial cut was deepened over a width considerably greater than that of the original shallow incision so that the experiments might be comparable in this respect, since the width of a deep adaxial incision has been shown to have an important organogenetic effect (Wardlaw, 1955*a*). In a few cases all four cuts were deepened; this did not affect the result obtained. Similarly, in the case of the control specimens, in which comparable primordia were isolated by deep cuts at the outset, some primordia were isolated by four deep cuts, on large or small plugs, and some by a single adaxial incision.

In earlier experiments on this species it was concluded that the determination of a primordium as a leaf normally coincides with the formation of an enlarged apical cell which projects from the surface of the meristem and is becoming lenticular in shape, characteristically orientated with respect to the shoot apex (Cutter, 1956). In most specimens the presence or absence of such a cell can be readily observed under a binocular microscope. Using this criterion, only primordia not yet determined as leaves were used in these experiments, except where otherwise stated; specimens in which there was any doubt as to the condition of the primordium to be isolated were rejected. Records were kept of the stage of development of the primordium at the time of the initial isolation, this being judged on an arbitrary basis. For convenience and to conform to earlier usage this has been called the stage of the plastochrone; however, this is not strictly correct, since P_2 or P_3 primordia may still be in the condition here designated as early or middle plastochrone. The terminology used in describing primordia and presumptive leaf sites is that of Snow and Snow (1931).

In some of the tables the proportion of the isolated primordia which developed as leaves is presented both as a percentage of the total number of primordia isolated, and as a percentage of those which responded to the stimulus of deepening the adaxial incision and developed as either leaves or buds ($L+B$). This was done because it was felt that at least two separate sets of factors were involved: factors stimulating a renewal of growth in the isolated primordia, and factors which determined their development either as



Text-figs. 1–4. Diagrams showing the position of the incisions.

Text-fig. 1. Four shallow incisions, plug large.

Text-fig. 2. Four shallow incisions, plug small.

Text-fig. 3. Isolation by four shallow incisions on a large plug, as in Text-fig. 1, after deepening the adaxial cut.

Text-fig. 4. Isolation by four shallow incisions on a small plug, as in Text-fig. 2, after deepening the adaxial cut.

ac = apical cell.

leaves or as buds. Statistical comparison was made between those specimens stimulated into renewed growth ($L+B$) and control specimens, or the results of earlier experiments with deep cuts (Cutter, 1954).

EXPERIMENTAL RESULTS

(i) *General analysis of experiments in which the adaxial incision was deepened*

The results of all experiments in which undetermined visible leaf primordia or presumptive leaf positions were isolated by four shallow cuts, on both large and small plugs, and the adaxial incision was subsequently deepened, are presented in Table I.

TABLE I

The Development of Primordia Isolated by Shallow Cuts with Subsequent Deepening of the Adaxial Cut

Primordium isolated	Total no. of isolations (T)	Result of treatment			Per cent.	
		No growth	Buds (B)	Leaves (L)	$\frac{L}{L+B}$	$\frac{L}{T}$
I_2	1	—	—	1	—	—
I_1	4	2	1	1	—	—
P_1	109	62	36	11	23·4	10·09
P_2	1	1	—	—	—	—
Total	115	65	37	13	26·0	11·3

It will be seen that, whereas in earlier experiments with shallow cuts in which the adaxial cut was not deepened the disappearing primordia eventually became quite invisible, and the tissue of the isolated plug became concave and gave rise to scales (Wardlaw and Cutter, 1956), in some specimens deepening of the adaxial cut had the effect of stimulating the residual isolated primordia into growth. In other specimens deepening the adaxial cut was without organogenetic effect, subsequent development of the isolated plugs being comparable with that of those in which the cut was not deepened. These results are recorded under the heading 'no growth', since the primordia continued to disappear and there was no outgrowth of tissue from the isolated plug in response to the deepening of the cut.

From the results presented in Table I, therefore, it is possible to conclude firstly (in accordance with inferences from earlier experiments), that the deepening of the adaxial cut may provide a stimulus to growth, since it may result in the outgrowth of growth centres which would otherwise have become totally dissipated, and secondly, that in many cases even the provision of such a stimulus fails to cause outgrowth of tissue; that is to say, that isolation by four shallow incisions can result in permanent loss of the capacity for growth of a primordium or growth centre.

Further appraisal of the results presented in Table I indicates that approximately one-quarter of those primordia which were stimulated into growth by deepening the adaxial incision developed as leaves. Since these primordia were not determined as leaves at the time of the initial isolation by shallow cuts, such a result was not to be expected on the basis of conclusions from earlier experiments with deep cuts, unless the process of leaf determination can proceed while the primordium is disappearing. (That such an explanation is not, in fact, possible, will be shown later.) Moreover, the results are in marked contrast with those obtained from control specimens in which comparable P_1 primordia were isolated by deep cuts at the outset. Of 21 specimens treated in this way, in 20 the isolated P_1 primordium developed as a bud, and in 1 it underwent no growth. No leaves were obtained. There is a statistically significant difference between the results obtained by isolating P_1 primordia with four shallow cuts and later deepening the adaxial cut (Table I), and those obtained by isolating comparable P_1 primordia by deep cuts at the outset. Those which responded to the stimulus of deepening the cut and underwent further growth ($L+B$ in Table I) were compared both with the 21 control specimens ($\chi^2 = 5{\cdot}5915$; for one degree of freedom, $p < 0{\cdot}02$), and with the results of earlier experiments (Cutter, 1954) with initial deep cuts ($\chi^2 = 8{\cdot}1049$; for one degree of freedom, $p < 0{\cdot}01$).

The organs included in column 5 of Table I under the general heading 'leaves' were, in fact, somewhat variable; while some were normally orientated dorsiventral leaf primordia, others consisted of variously orientated double or triple leaves, and yet others were organs of radial symmetry. In every instance, however, the vascular system of the isolated organ was that of a leaf. In some of these specimens there was a subsequent development of lateral or axillary meristems. These various developments will be described in more detail later (Table VIII); but at present it is cogent to consider further data which might throw light on the possible causes of their development as leaves.

(ii) *Effect of the stage of development of the primordium*

In earlier experiments on the isolation of P_1 primordia by deep cuts a clear correlation was found between the stage of development, or plastochrone, attained by the primordium at the time of isolation, and its subsequent development, a higher proportion of primordia isolated in late plastochrone developing as leaves (Cutter, 1954). For purposes of comparison, these data are reproduced here in Table II above.

TABLE II

The Effect of the Stage of the Plastochrone Attained at the Time of Isolation on the Subsequent Development of P_1 Primordia Isolated by Deep Cuts on Large Plugs of Tissue (1954)

Stage of plastochrone	Total no. of isolations	Result of treatment		$\% \frac{\text{Leaves}}{\text{Total isolations}}$
		Buds	Leaves	
Early	37	36	1	2·7
Middle	58	56	2	3·4
Late	25	19	6	24·0
Total	120	111	9	7·5

No such clear correlation can be shown with primordia originally isolated by shallow cuts with subsequent deepening of the adaxial incision (Table III). A comparison of Tables I and III with Table II also shows the higher proportion of primordia which developed as leaves in the experiments now reported.

TABLE III

The Effect of the Stage of the Plastochrone Attained at the Time of Isolation of P_1 Primordia by Shallow Cuts on their Development after Subsequent Deepening of the Adaxial Cut

Stage of plastochrone	Total no. of isolations (T)	Result of treatment			Per cent.	
		No growth	Buds (B)	Leaves (L)	$\frac{L}{L+B}$	$\frac{L}{T}$
Early . .	73	47	21	5	19·23	6·85
Middle . .	28	13	10	5	33·33	17·86
Late . .	8	2	5	1	16·6	12·5

(iii) *Effect of the length of time between initial isolation and subsequent deepening of the adaxial incision*

The period of time which elapsed between the initial isolation of the primordium by shallow incisions and the subsequent deepening of the adaxial cut was somewhat variable, due in large part to the fact that the isolated primordia took varying periods of time to reach the point of disappearance. Since it was considered that there might be some correlation between this period of time and the development of the isolated primordia, the results were analysed on this basis (Table IV).

TABLE IV

The Effect of the Length of Time between Initial Isolation by Shallow Cuts and Subsequent Deepening of the Adaxial Cut on the Development of Isolated P_1 Primordia

Development of P_1	Total no.	No. of days between initial isolation and subsequent deepening of the adaxial cut	
		Range	Mean ± S.E.
No growth	62	15–84	30·82 ± 1·59
Buds	36	19–84	37·0 ± 2·67
Leaves	11	13–106	29·55 ± 7·97

The considerable range in the number of days which elapsed between the original isolation and the deepening of the cut gives an indication of the variability in the speed of disappearance of the isolated primordia. While it is certainly true to say that by allowing a sufficient length of time to elapse before deepening the cut it would be possible to obtain the result 'no growth' at will, it may be noted that in fact there was no significant difference in this respect between the three groups of specimens. Thus the length of this period of time does not seem to be important in determining the development of the isolated primordia.

(iv) *Effect of the rate of growth of the apex between initial isolation and subsequent deepening of the adaxial incision*

Both the amount and the rate of growth of the specimens during the time between the initial and subsequent incisions were variable. There was no evident correlation between the number of new primordia formed during this time and the development of the isolated primordia (Table V).

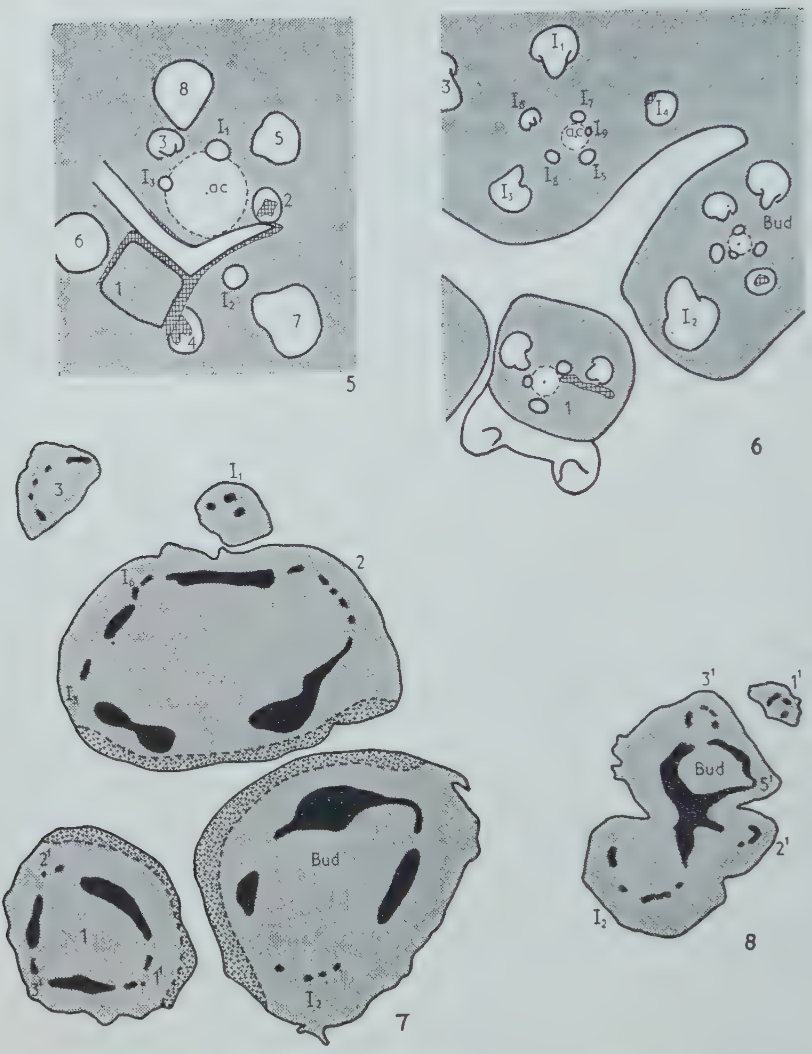
TABLE V

The Effect of the Number of New Primordia Formed and the Rate of Growth of the Shoot Apex between Initial Isolation by Shallow Cuts and Subsequent Deepening of the Adaxial Cut on the Development of Isolated P_1 Primordia

		No. of new primordia between initial isolation and subsequent deepening of the adaxial cut		Plastochrone (days)	
Development of P_1	Total no.	Range	Mean±S.E.	Range	Mean±S.E.
No growth . .	62	0–7	1·29±0·21	5·0–98	24·77±2·41
Buds . . .	36	0–7	2·14±0·25	5·5–42	21·45±2·06
Leaves . .	11	0–3	1·27±0·27	6·5–53	22·82±4·06

The rate of growth of the apices as indicated by the length of the plastochrone is also included in Table V; it can be seen that there is apparently no significant difference in this respect between the groups of specimens. It should be pointed out, however, that owing to the small number of primordia formed in many specimens during the period in question this method of presenting the rate of growth is only very approximate. For example, if a period of 42 days elapsed between initial isolation of the primordium and subsequent deepening of the cut and only one new primordium was formed on the apex during that time, the plastochrone interval was taken as 42 days. However, the inception of a second primordium might have been just about to take place at this time; the margin of error is therefore considerable, but may be expected to be relatively uniform for each of the groups of specimens.

It is important to note that in one specimen an isolated primordium developed as a bud when seven new primordia were present at the time of deepening the cut; that is, at the time of making the deep incision the disappearing P_1 had attained the position of P_8. In five specimens four new primordia were present at the time of deepening the cut, and in one of these I_2, which could be seen to possess a lenticular apical cell, was also isolated by a deep cut at the same time as the disappearing P_1. While P_1 became a bud, I_2, a primordium actually two plastochrones younger, developed as a leaf, with a bud lateral to it (Text-figs. 5–8). Findings such as these indicate that the process of leaf determination cannot continue when the primordium has ceased growth as such and is disappearing; that is, proximity of an inactive growth centre to the shoot apex over a period of time is not sufficient to result in its determination as a leaf. Isolation by shallow incisions does not mechanically preclude



TEXT-FIGS. 5–8. A specimen in which P_1 was isolated by four shallow cuts and allowed to regress until it was hardly visible at all. The adaxial cut was then deepened, and at the same time I_2, which could be seen to possess a lenticular apical cell, was also isolated by a deep adaxial cut.

Text-fig. 5. Surface view of the apex 41 days after the initial isolation, 8 days after deepening the adaxial cut. There is little evidence of growth on the P_1 plug; I_2 is developing as a leaf. (× 12.)

Text-fig. 6. Thirty-eight days later. A bud with six leaf primordia has developed on the P_1 plug. A bud with seven leaf primordia has developed in a position lateral to I_2, which has become orientated towards it to some extent. The sub-apical region is stippled. Necrosed tissue cross-hatched; *ac* = apical cell. (× 12.)

Text-fig. 7. Transverse section of the specimen.

Text-fig. 8. Higher transverse section of I_2 and the lateral bud, showing the orientation of I_2 towards it. Prestelar tissue black; necrosed tissue dotted. (× 10.)

determination, since when apices are in active growth primordia thus isolated usually continue to grow and develop as leaves (Wardlaw and Cutter, 1956).

(v) *Effect of the size of the isolated plug*

A comparison of the results of those experiments in which the original shallow incisions were made close to the primordium, resulting in its isolation on a small plug of the type shown in Text-fig. 2, with those isolated on large plugs (Text-fig. 1) is presented in Table VI. It is noteworthy that about twice as many primordia isolated on small plugs failed to respond to the stimulus of deepening the adaxial cut and underwent no further growth. Since the adaxial incision was deepened over a comparable width in both types of experiment (Text-figs. 3 and 4), this result was presumably due to the proximity to the growth centre of the original shallow incisions. Approximately the same mean length of time had elapsed before deepening the cut in both groups of experiments (large plugs, 32·45±3·40 days: small plugs, 30·05±1·72 days).

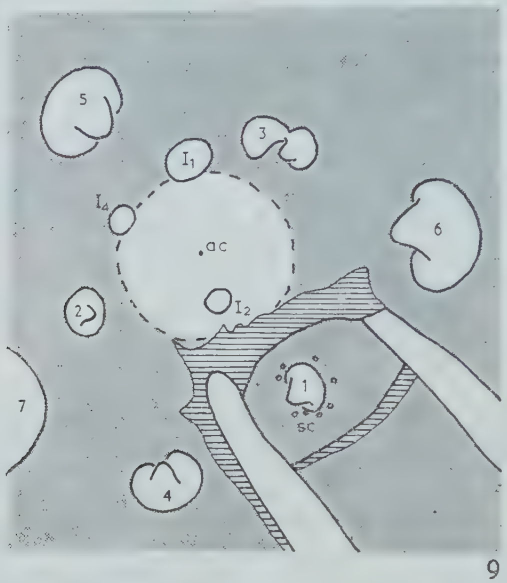
TABLE VI

The Effect of the Size of the Plug of Tissue on the Development of P_1 Primordia Isolated by Shallow Cuts with Subsequent Deepening of the Adaxial Cut

Size of plug	Total no. of isolations	Result of treatment: No growth	Buds	Leaves	% no growth / Total
Large . .	53	20	25	8	37·73
Small . .	56	42	11	3	75·0

(vi) *Effect of the position of the deepened incision*

Since Wardlaw (1949*b*) had shown that the growth of young leaf primordia can be enhanced by isolating them from adjacent primordia by deep radial cuts, it became of interest to discover whether a similar stimulation of growth in the residual primordia could be brought about by deepening any of the cuts other than the adaxial one. Comparable experiments were therefore carried out in which the abaxial or radial shallow incisions were subsequently deepened instead of the adaxial one. Deepening the abaxial incision (3 specimens) did not result in any stimulation of growth. This was to be expected, since initial isolation by a deep abaxial incision is also without effect on the development of the primordium (Wardlaw and Cutter, 1956). The results of deepening both radial cuts are available for 22 specimens, in only one of which stimulation of growth was obtained. In this specimen the isolated P_1 developed as a dorsiventral, circinate leaf primordium of approximately normal size, orientated towards P_4 (Text-fig. 9). These experiments therefore show that stimulation of renewed growth may be brought about by deepening incisions other than the adaxial one, although this treatment is apparently the most effective, and also afford additional evidence of the difference between isolation by shallow incisions which are subsequently deepened and isolation by cuts which are deep at the outset, since primordia isolated by deep radial incisions by Wardlaw (1949*b*, 1950), and others used as controls in the present experiments, were normally orientated towards the shoot apex. Similar results were obtained in the potato (Sussex, 1955). Problems of orientation are discussed in greater detail later.



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TEXT-FIG. 9. An apex in which P_1 was isolated by four shallow cuts and allowed to disappear, both radial cuts then being deepened. P_1 has been stimulated to grow again and has developed as a dorsiventral leaf primordium of approximately normal size, orientated towards P_4. P_3 is a double leaf primordium. I_2 is displaced and I_3 has failed to develop at this stage.

The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell. (×18.)

(vii) *Isolation of older primordia determined as leaves*

Isolation, by four shallow cuts, of primordia which were either just determined as leaves or just not determined, was undertaken to see if in fact such primordia would regress or disappear, and, if so, how they would develop after deepening the cut. Fifteen primordia in this transitional stage were therefore isolated on 11 other specimens, two consecutive primordia sometimes being treated on one apex. The results are in agreement with the small number of isolations of such primordia previously carried out (Wardlaw and Cutter, 1956), and indicate that, in general, isolation by four shallow cuts does not cause the disappearance of primordia already determined as leaves (Table VII). Both the primordia which did disappear, and a few of the others, were recorded as being only probably determined at the time of isolation. In the specimen in which P_4 disappeared, the adaxial incision was later deepened and a bud was obtained.

These experiments therefore provide additional evidence that those primordia which did regress after isolation by shallow incisions but developed as leaf primordia after the stimulus of subsequent deepening of the adaxial cut (Table I) were not determined as leaves at the time of the initial isolation.

TABLE VII

The Development of Primordia determined or almost determined at the Time of Isolation, after Isolation by four Shallow Cuts

Primordium isolated	Total no. of isolations	No. which disappeared	No. which developed as normal leaf primordia
P_1	2	1	1
P_2	7	—	7
P_3	4	—	4
P_4	2	1	1

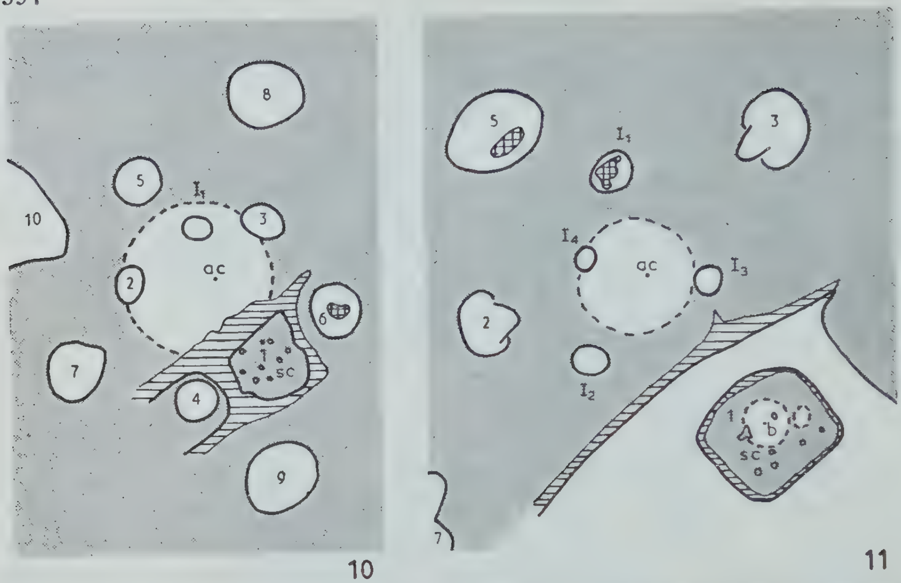
MORPHOLOGICAL AND ANATOMICAL OBSERVATIONS

No clear correlation could be established between the external appearance of the isolated primordium at the time of deepening the adaxial incision and its subsequent development. Primordia which, although they had diminished in size, were still visible at the time of deepening the cut, sometimes continued to disappear and scales arose at their position; conversely, in several instances it was recorded that one or more incipient scales were arising near or upon the site of P_1 at the time of deepening the cut, and yet after the cut was deepened the isolated P_1 developed as a bud bearing the scale upon its surface. An extreme case of this, in which numerous incipient scales were present, is illustrated in Text-figs. 10 and 11. It is noteworthy that the scales were always present on the bud in the same position with respect to its apex as they had been relative to the centre of the disappearing P_1. The conclusion that the organ stimulated to develop by deepening the adaxial cut was indeed derived from the growth centre initially isolated is supported by a few experiments in which the isolated primordia were marked with indian ink.

These observations suggest that the external appearance of such isolated areas of tissue is not always a precisely reliable indication of their metabolic condition. The cellular structure of the surface layer of the isolated plugs at the time of deepening the adaxial cut could be observed with varying degrees of clarity with a binocular microscope; no cell corresponding to the enlarging lenticular apical cell of a leaf primordium was ever observed. In previous histological examination of plugs isolated by shallow incisions (Wardlaw and Cutter, 1956) such cells were also not seen.

In specimens in which the isolated primordia underwent no further growth after deepening the adaxial cut, the isolated plugs remained flat or became more or less concave, giving rise to numerous scales over the whole surface. No vascular traces were associated with the vanished primordium (see Wardlaw and Cutter, 1956, Text-figs. 29 and 30), but prestelar tissue pertaining to the shoot apex, present at the time of isolation, passed across the isolated plug (Text-fig. 12).

Isolated primordia which developed as buds after deepening the adaxial incision varied considerably in size, presumably depending upon the amount

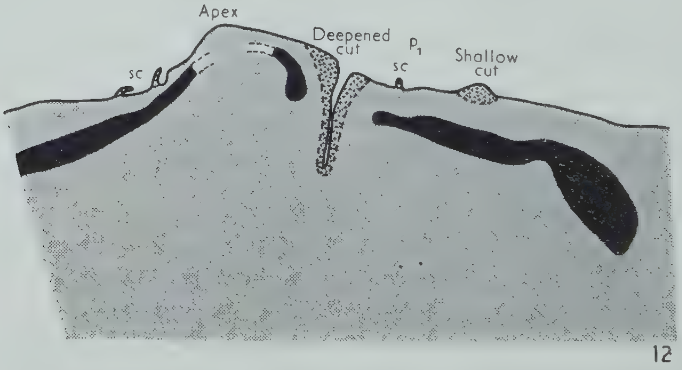


TEXT-FIGS. 10 and 11. A specimen in which P_1, isolated by four shallow cuts with subsequent deepening of all four incisions, developed as a small bud bearing incipient scales on its surface.

Text-fig. 10. At the time of deepening the cuts, 19 days after the initial isolation by shallow incisions. Incipient scales (*sc*) are present around the position of P_1, which is still just visible but appears to have an incipient scale centrally upon it.

Text-fig. 11. Twenty days later. A small bud *b* with a scale close to its tip is present on the P_1 plug, with a leaf primordium abaxially towards P_6. Subsequently a second leaf primordium was formed towards P_4.

The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell. (×18.)

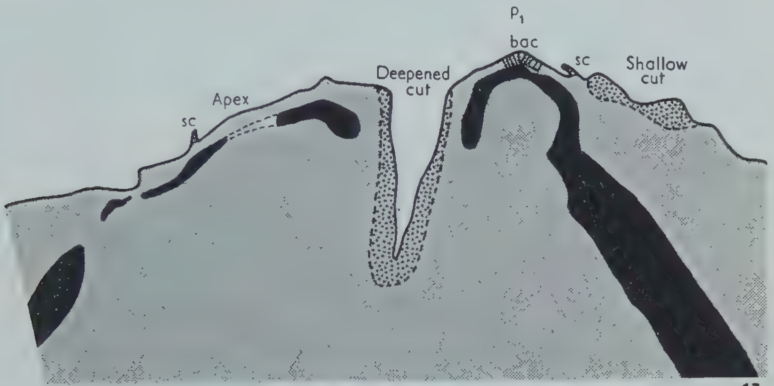


TEXT-FIG. 12. Longitudinal section of a specimen in which P_1, isolated by four shallow cuts on a small plug with subsequent deepening of the adaxial incision, disappeared and did not react to the stimulus of deepening the cut. A scale (*sc*) is present approximately at the position of P_1. No leaf traces are associated with P_1, but axial prestelar tissue passes across the isolated plug. Note the displacement and tilting of the shoot apex. Compare Text-fig. 13.

Prestelar tissue black; indistinct prestelar tissue outlined with a broken line; necrosed tissue dotted; *sc* = scales. (×20.)

of meristematic tissue remaining. Their vascular system was solenostelic, and made contact with axial vascular tissue abaxially and laterally, as could be seen in both transverse and longitudinal section (Text-fig. 13). Growth of these buds was comparatively rapid, and this supports an earlier suggestion that the retardation or cessation of growth in organs isolated by four deep cuts, which have no connexion with axial stelar tissue, might be due to inadequate transmission of nutrients (Cutter, 1956).

In those specimens in which the isolated primordia developed as leaves, meristematic growth was usually localized and restricted, approximately, to the area of the isolated P_1 itself (Pl. XII, Figs. 1 and 3; Text-figs. 14–28). Incipient scales could usually be observed outlining the position of the primordium, and scales were often present over the rest of the area of the isolated plug. (Scales are not always indicated in the Text-figures.) In some specimens no vertical growth took place over the rest of the plug; in others, there was some outgrowth of surrounding tissue, which bore scales. Scales also surrounded the isolated primordia, as can be seen in some of the transverse sections. Cumulatively the observations show that the area of the isolated plug which retained its potentialities for meristematic growth for the longest time was that of the original growth centre.



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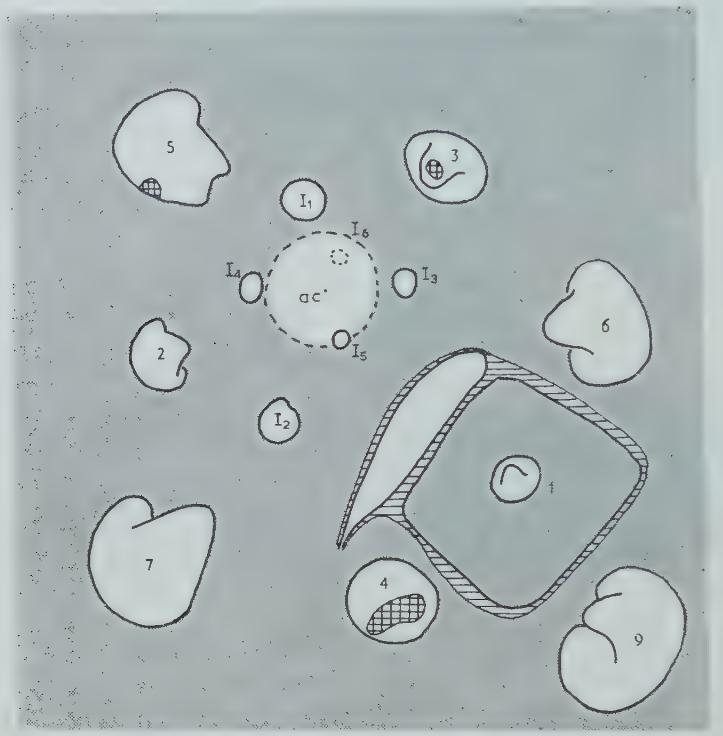
TEXT-FIG. 13. Longitudinal section of a specimen in which P_1, isolated by four shallow incisions on a small plug with subsequent deepening of the adaxial cut, developed as a bud. The section does not pass medianly through the main shoot apex, which is tilted, as in Text-fig. 12. The vascular system of the P_1 bud is solenostelic and connects abaxially with axial vascular tissue. Compare Text-fig. 12. The abaxial shallow cut has opened out.

Prestelar tissue black; indistinct prestelar tissue outlined with a broken line; necrosed tissue dotted; *sc* = scales; *bac* = bud apical cell. (×20.)

As stated earlier, a considerable variety of leaf developments were obtained in these experiments. The various developments are presented in summary form in Table VIII. It may be noted that both the symmetry and orientation of these organs were variable. Dorsiventral leaves normally orientated towards the shoot apex (Pl. XII, Figs. 1 and 2 and Text-figs. 14, 16, and 19) were obtained, and these had normal leaf vascular structure, except for a continuous strand of prestelar tissue in an adaxial position. This consisted of axial vascular tissue isolated by the deepened cut. Dorsiventral leaves which were orientated in various directions, e.g. towards P_4 (Text-figs. 20–22), or obliquely towards the adaxial P_6 corner of the plug (Text-figs. 23–26), were also obtained. In some instances leaf primordia which ultimately became dorsiventral and circinate passed through a phase of approximately radial symmetry; this was sometimes observable externally, during the weekly observations under a binocular microscope (Text-figs. 18, 21, and 24), but in other specimens it was only detected with certainty subsequently, from observations of transverse sections (Pl. XIV, Figs. 5 and 6). In two cases leaf primordia of apparently true radial symmetry developed, and these did not become dorsiventral during the period of observation (Pl. XIII, Fig. 3 and Text-figs. 27 and 28). The vascular system of these radial and approximately radial leaf primordia and of those dorsiventral primordia which passed through a phase of radial symmetry was characterized by a complete ring of discrete strands of prestelar tissue (Pl. XIV, Figs. 7–9 and Text-figs. 29–33) in contrast to the strands in the form of a horse-shoe, open on the adaxial side, which typify a normal dorsiventral leaf primordium of this species. Near the apex of such a radially symmetrical primordium, and in some instances at



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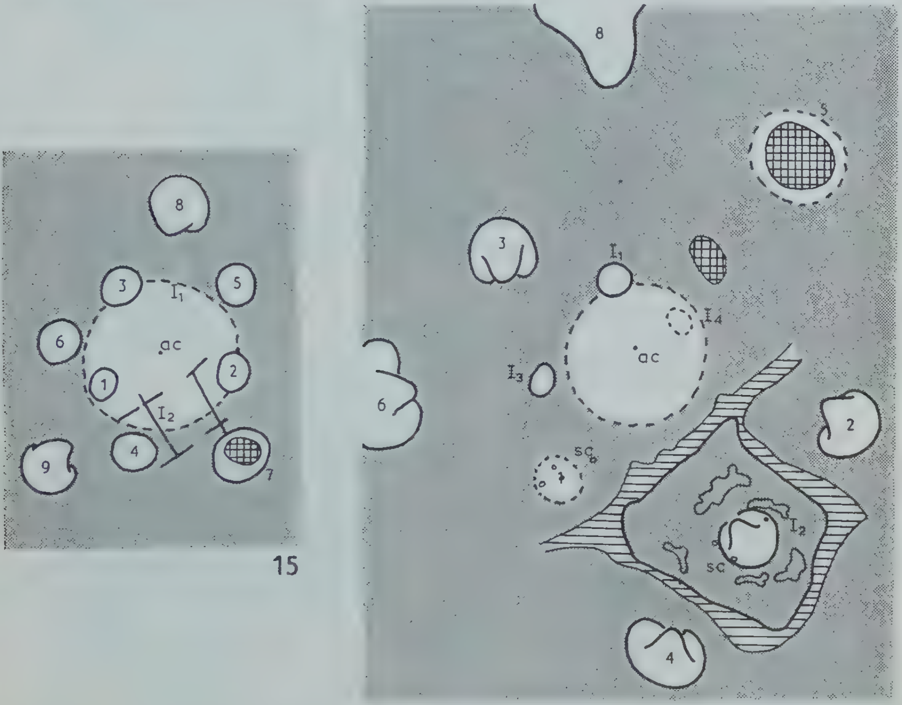
TEXT-FIG. 14. An apex in which P_1, isolated by four shallow cuts with subsequent deepening of the adaxial incision, has developed as a normally orientated dorsiventral leaf primordium which is perhaps slightly smaller than normal. Compare Pl. XII, Fig. 1.

The sub-apical region is stippled; necrosed tissue cross-hatched; *ac* = apical cell. (× 18.)

lower levels also, these strands were more or less joined to form a solenostele (Pl. XIV, Figs. 5 and 6).

Double leaf primordia, variously orientated, were also obtained, and in one instance three small, discrete, dorsiventral leaf primordia developed on the isolated P_1 plug, in a position which appeared somewhat more abaxial than that of P_1 itself (Pl. XIII, Fig. 4; Text-fig. 34). These primordia were all orientated towards a central scaly hollow. Transverse sections confirmed the external observations, and also established the absence of any bud on the plug (Text-figs. 35–40). At a lower level, the vascular tissue of the three primordia came to form an ellipse composed of numerous discrete strands of prestelar tissue, and at a still lower level the vascular structure was similar to that of a single isolated leaf primordium (Text-figs. 35 and 36).



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TEXT-FIGS. 15 AND 16. A specimen in which the presumptive I_2 position, isolated by four shallow incisions (two of which were not quite continuous) with subsequent deepening of the adaxial cut, developed as a normally orientated dorsiventral leaf primordium of approximately normal size.

Text-fig. 15. At the time of the initial isolation of the I_2 position by shallow cuts.

Text-fig. 16. Fifty-seven days after deepening the adaxial incision, 11 weeks after the initial isolation. I_2 is now a normal dorsiventral leaf primordium; scales (*sc*) are present over the rest of the plug. Compare Pl. XII, Fig. 2.

P_1 has become somewhat scaly and is indistinct; I_1 suffered some initial damage and is smaller than normal.

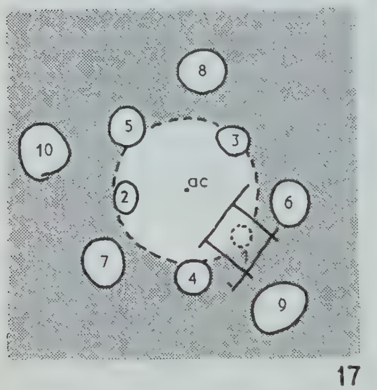
The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell. (×18.)

TABLE VIII

Analysis of Primordia which Developed as Leaves after Isolation by Shallow Cuts with Subsequent Deepening of the Adaxial Cut

Primordium isolated	No. of such primordia	Organ developed	Symmetry*	Orientated towards	Presence of axillary or lateral meristems
I_2	1	Leaf	D	Apex	—
I_1	1	Double leaf	D(R)	Abaxial P_3 corner	—
P_1	1	Leaf	R	—	—
	1	Leaf	R	—	+
	1	Leaf	D	Apex	—
	2	Leaf	D(R)	Apex	—
	2	Leaf	D(R)	P_4	+
	1	Leaf	D	P_4	+
	1	Leaf	D(R)	Adaxial P_6 corner	—
	1	Double leaf	D(R)	Adaxial P_4 corner	+
	1	Three leaves	D	Apex, P_4 and P_6	—

* D = Dorsiventral; R = Radial; D(R) = Dorsiventral, with a phase of approximately radial symmetry. This phase was not always recognized from external observations, but was evident from sectioned material.



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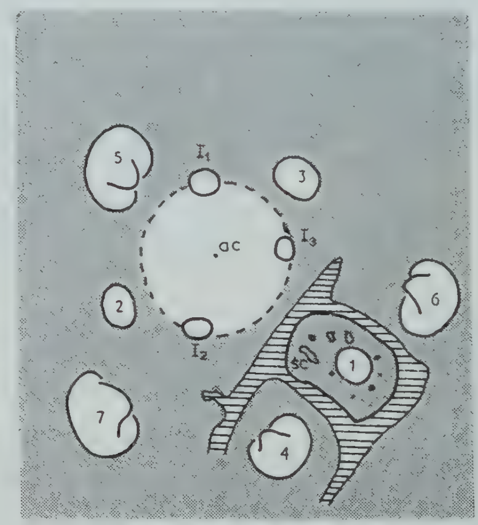

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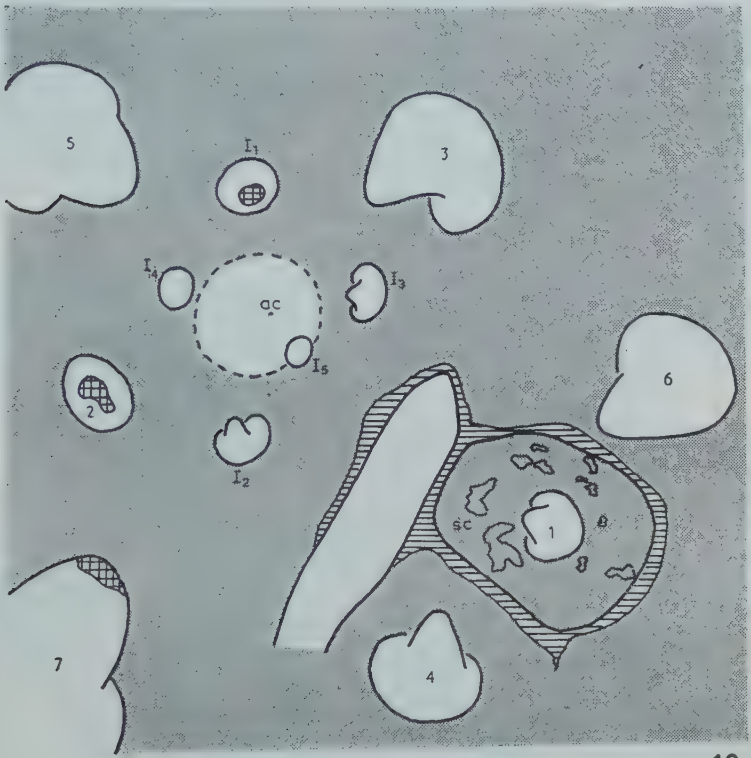
TEXT-FIGS. 17–19. A specimen in which P_1, isolated by four shallow incisions with subsequent deepening of the adaxial cut, developed as a normally orientated dorsiventral leaf primordium in approximately normal size relationship with other primordia. Scale bases (*sc*) are present over the rest of the P_1 plug. A comparison of Text-fig. 17 with Text-figs. 18 and 19 indicates the progressive radial elongation of the isolated plug which took place. Transverse sections indicated that the primordium probably passed through a phase of approximately radial development.

Text-fig. 17. At the time of isolation of P_1 by shallow incisions.

Text-fig. 18. Forty-five days later, 25 days after deepening the adaxial incision. P_1 is developing as a localized growth, evidently a leaf of normal size; its symmetry is not yet certain. Scales (*sc*) are present over the rest of the plug. (× 18.)

As Table VIII shows, in some specimens small meristems developed on the plug in positions lateral or axillary to the isolated primordium (Text-figs. 22, 28, and 31–33; Pl. XIV, Figs. 8 and 9). In most instances, these meristems were clearly buds, which quickly formed leaf primordia, but in a few cases the first-formed meristems appeared to be small leaf primordia, usually orientated towards the isolated primordium. These meristems presumably developed from any residual meristematic tissue on the plug, between scaly parenchymatous tissue. Although isolated P_1 primordia sometimes seemed to be orientated towards these small buds (Text-figs. 22, 31–33), development of such lateral or axillary meristems was always belated, and it seems rather unlikely that at such a late stage they could affect the orientation of primordia so much older. This is, however, possible; for the orientation of a young leaf primordium towards a bud which develops near it as a result of isolation from or damage to the parent shoot apex has been noted on several occasions (Text-figs. 5–8; see also Wardlaw and Cutter, 1955). However, in such cases the leaf primordia and buds are usually of more uniform age.



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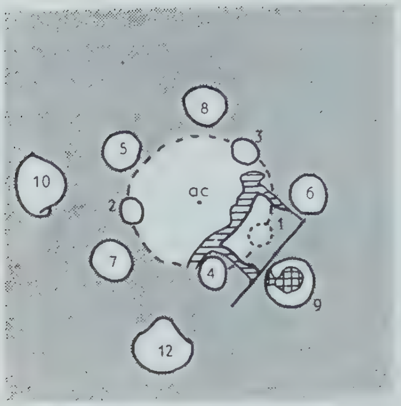
Text-fig. 19. Nineteen days later. P_1 is now a normally orientated dorsiventral leaf primordium.

The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell. (× 18.)



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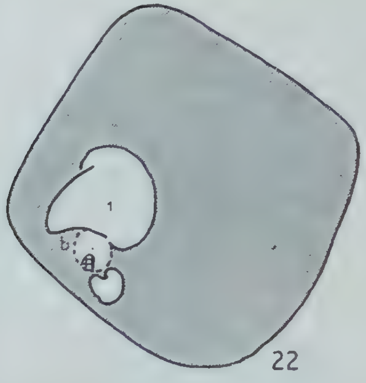

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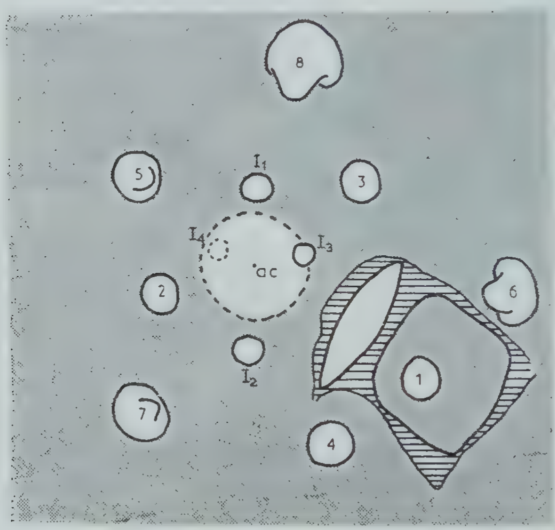

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TEXT-FIGS. 20–22. An apex in which P_1, isolated by four shallow cuts with subsequent deepening of the adaxial incision, developed as a leaf primordium which was initially of approximately radial symmetry but eventually became orientated either towards P_4 or to the lateral bud which developed on the plug. See Text-figs. 29–33.

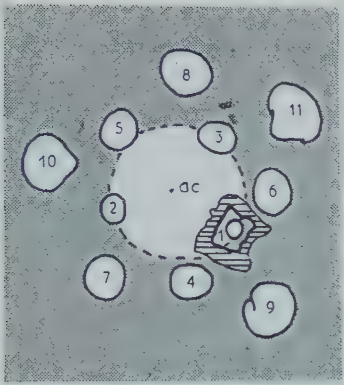
Text-fig. 20. At the time of deepening the adaxial incision, 13 days after the initial isolation by shallow cuts. P_1 was just about visible.

Text-fig. 21. Forty-three days later. P_1 is a leaf primordium of approximately normal size and radial symmetry, with incipient scales (not shown) around it.

Text-fig. 22. Thirty-two days later. (The isolated P_1 plug only is shown, in the same orientation as before.) P_1 has now become orientated towards P_4 or to the small bud (*b*) which has developed on the plug.

The sub-apical region is stippled; necrosed tissue cross-hatched; *ac* = apical cell. (× 18.)

TEXT-FIGS. 23–26. An apex in which P_1, isolated by four shallow cuts with subsequent deepening of the adaxial incision, developed as a dorsiventral leaf primordium orientated towards the adaxial P_6 corner of the plug.



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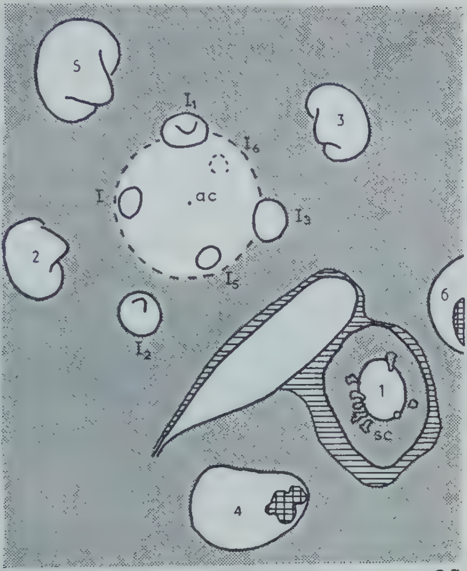

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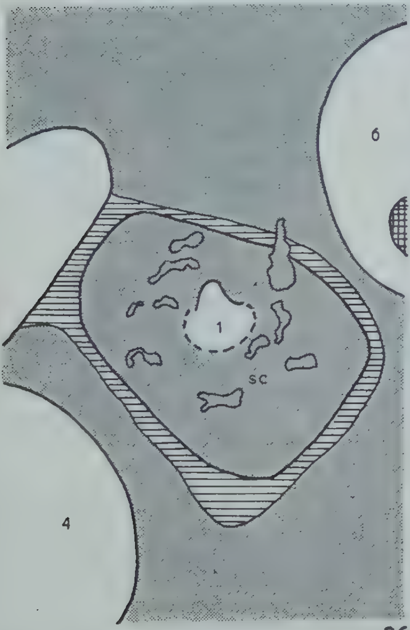

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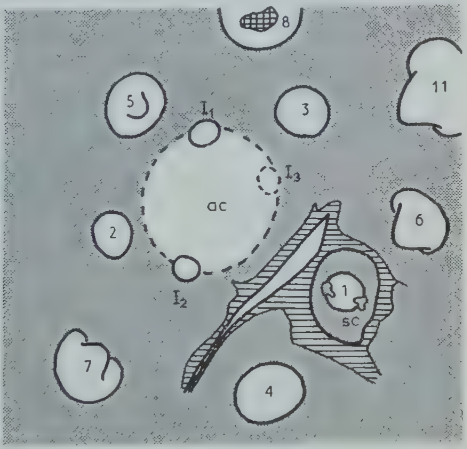

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Text-fig. 23. Twenty days after isolation by shallow incisions P_1 is still quite conspicuous.

Text-fig. 24. A month after deepening the adaxial cut, which was carried out 106 days after the initial isolation, when P_1 was still visible but only of about the size of I_2. Slight growth of the central region of the plug is now taking place, with a single scale both adaxial and abaxial to it.

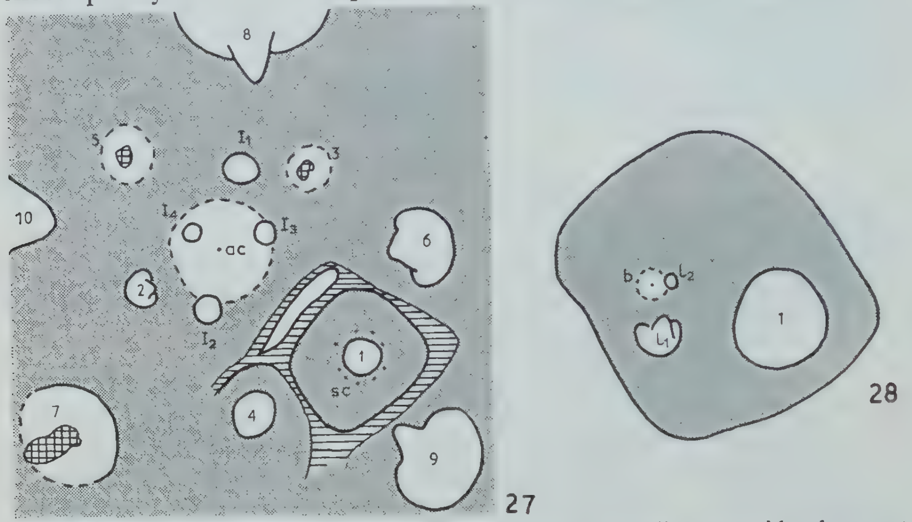
Text-fig. 25. Sixteen days later. A swelling of approximately normal size is present on the P_1 plug; it is somewhat radially elongated but less evidently dorsiventral and circinate than I_3. Scales (*sc*) are arising upon the basal parts of the swelling. The isolated plug is now slightly elongated in a radial direction.

Text-fig. 26. Thirteen days later, 24 weeks after the initial isolation by shallow incisions and 9 weeks after deepening the cut. P_1 has now developed as a dorsiventral leaf primordium of about the same size as I_3, orientated somewhat adaxially towards P_6. The rest of the tissue of the plug forms a scaly outgrowth on which the leaf is situated; the plug is now radially elongated.

The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell. (×18.)

DISCUSSION

The majority of the results of these experiments have been in accordance with expectations based on earlier findings, but in a number of specimens unexpected developments of considerable interest were obtained; these, in particular, merit discussion. The most notable new finding is that in *Dryopteris aristata* it is sometimes possible for a primordium not yet determined as a foliar organ to develop as a leaf primordium even when it is isolated from the shoot apex by a wide and deep adaxial incision. These primordia were certainly not yet determined when the original shallow incisions were made, for leaf primordia have been obtained not only from P_1 primordia but also from the similarly isolated presumptive positions of I_1 and I_2 (Table I). Although this result cannot yet be obtained at will, it nevertheless has occurred with some frequency in these experiments; since, with such undetermined primordia, there is no evident relationship between the development of an isolated primordium as a leaf and (*a*) its stage of development at the time of the initial isolation, (*b*) the time which elapsed between this isolation and the subsequent deepening of the adaxial cut, or (*c*) the rate of growth of the apex during that time, some explanation of the results must be sought on other grounds.



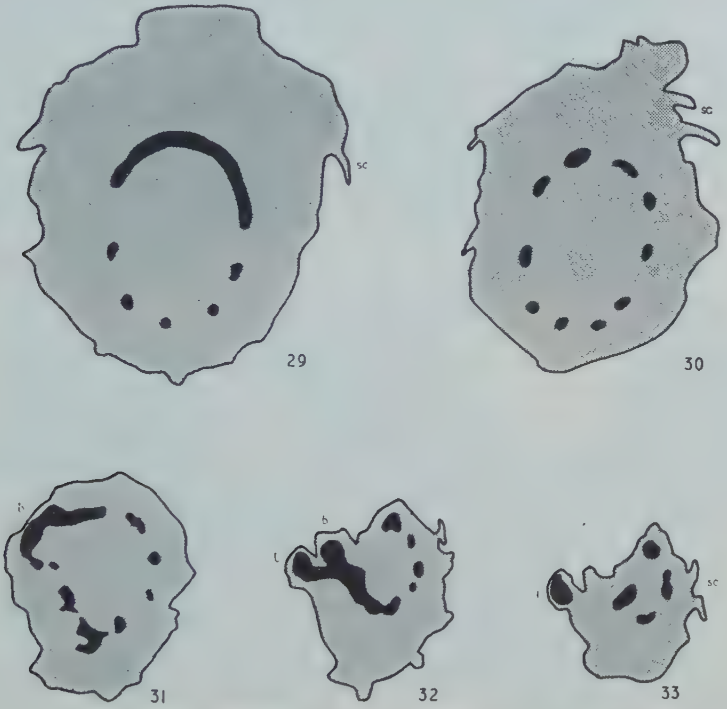
TEXT-FIGS. 27 AND 28. A specimen in which P_1, isolated by four shallow cuts with subsequent deepening of the adaxial incision, developed as a leaf primordium of radial symmetry. A small bud subsequently arose upon the plug in a lateral position. See also Pl. XIV, Figs. 3, 8, and 9.

Text-fig. 27. Twenty-one days after deepening the adaxial incision, 56 days after the initial isolation by shallow cuts. P_1 is a leaf primordium of approximately normal size, apparently of radial symmetry. Scales (*sc*) are present around it. Compare Pl. XIII, Fig. 3.

Text-fig. 28. The isolated plug only, similarly orientated, 32 days later. P_1 is a conical, approximately radially symmetrical organ. A small bud (*b*) has just developed on the plug; its first leaf primordium (l_1) was observed before the bud meristem.

The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell; *b* = bud; l_1, l_2 = the first two primordia of the bud. (× 18.)

In plants little is yet known about determination, i.e. the fixation of the path of development of an organ or tissue; nevertheless, this process is of great importance in morphogenesis. It is possible that *D. aristata* is particularly favourable material for such investigations; for, if isolated from the shoot apex by a wide and deep tangential incision, both prospective primordium sites and certain young visible primordia will develop, not as leaves, but as buds (Wardlaw, 1949*b*; Cutter, 1954, 1956). In this species, leaf and bud primordia are thus homologous. Leaf determination normally coincides with the formation of an enlarged, lenticular apical cell, and it seems likely that the factors responsible for the determination of a primordium as a leaf are normally extrinsic to the primordium itself. The fact that leaves are always found in characteristic relationship with, and orientation towards, a shoot apex, and never normally occur in its absence, supports the hypothesis that some regulative effect of the apex determines the development of a primordium as a leaf and prevents its alternative development as a bud. It is salutary at this point



TEXT-FIGS. 29–33. Acropetal transverse sections of the specimen illustrated in Text-figs. 20–22, in which P_1, isolated as described, developed as a leaf primordium which was initially of approximately radial symmetry but ultimately became orientated towards P_4 or the small bud which developed laterally on the plug. Sections of the P_1 plug only are shown here.

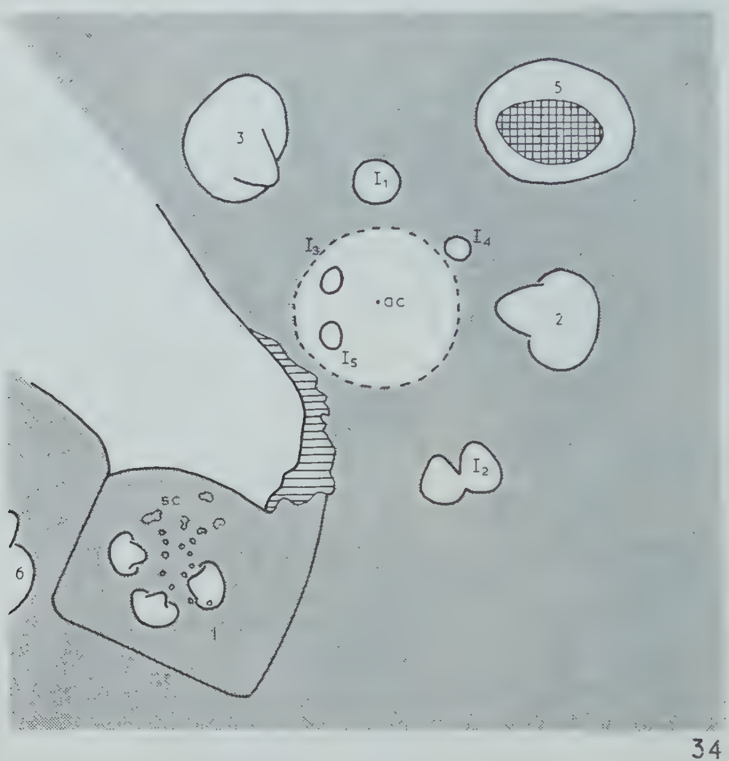
Text-figs. 29 and 30. In contrast to the normal vascular traces in the form of a horse-shoe, here they form a complete ellipse, a continuous strand of prestelar tissue being present adaxially in the basal regions.

Text-figs. 31–33. The prestelar tissue of the lateral bud (*b*) and its first leaf primordium (*l*) is now evident. In Text-fig. 33 the somewhat lateral orientation of P_1 towards P_4 can be seen (the shoot apex is towards the top of the page in all figures; P_4 is to the left).

Prestelar tissue black; *b* = lateral bud; *l* = the first leaf primordium of the bud; *sc* = scales. (×20.)

to recall the statements of Spemann (1938) and Wigglesworth (1954) that determination is not merely a restriction of the possible fates, but is also an evocation of inherent potentialities.

The present experiments establish two new facts which have a bearing upon the phenomenon of determination. Firstly, since disappearing primordia which have reached the position of P_5, for example, at the time of deepening the adaxial cut, may develop as buds, it is clear that to effect its determination as a leaf it is not sufficient for a piece of tissue simply to remain in contact with the shoot apex for the requisite length of time. It seems, therefore, that determination only occurs when the tissue has attained some particular state of competency, and that in leaf primordia of *D. aristata* this implies, at least, active and continuous vertical growth of the growth centre.[1] The second fact is



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TEXT-FIG. 34. A specimen in which three small dorsiventral leaf primordia were formed on the plug after isolation of P_1 by four shallow cuts with subsequent deepening of the adaxial incision. They are orientated towards a central scaly hollow. I_2 is a double primordium. Forty-two days after deepening the adaxial cut, 66 days after the initial isolation by shallow cuts. Compare Pl. XIII, Fig. 4; see also Text-figs. 35–40.

The sub-apical region is stippled; necrosed tissue cross-hatched; *sc* = scales; *ac* = apical cell. (× 18.)

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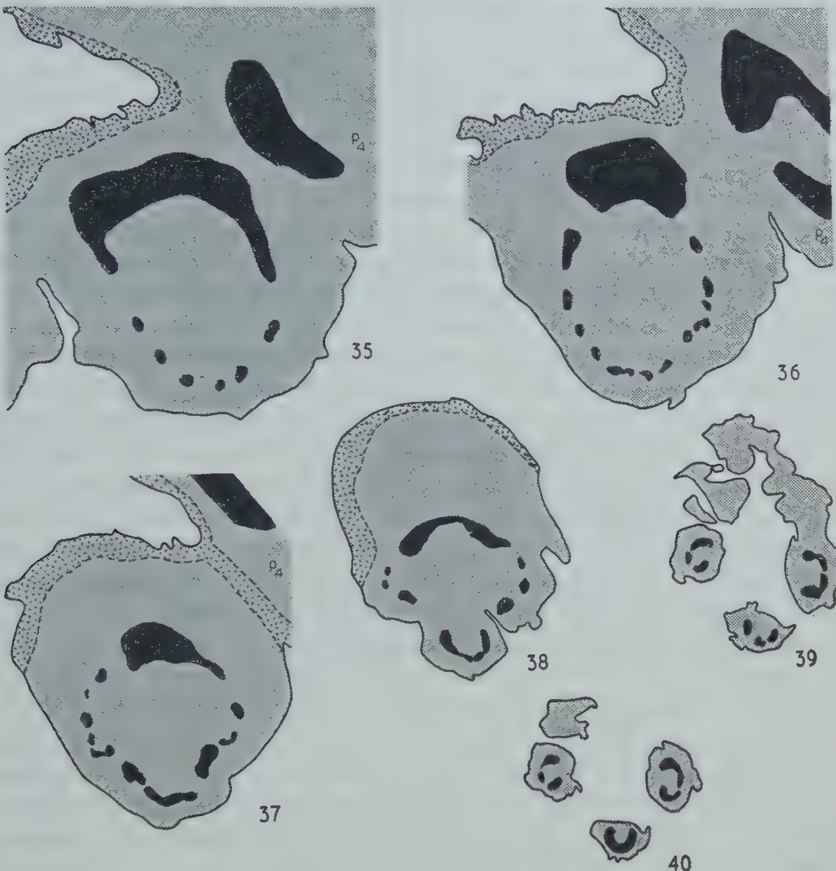
[1] This view is in accordance with the conclusions of zoologists also; for example, in some metamorphosing insects '. . . such organs must reach a certain size before they become "competent" to undergo metamorphosis' (Wigglesworth, 1954, p. 96).

that it is sometimes possible for an undetermined primordium to develop as a leaf even when isolated from the shoot apex by a wide and deep incision, indicating that there are circumstances in which leaf determination may take place without maintaining contact with the apex through the prestelar tissue. The association of leaf primordia with a shoot apex, however, is so invariable that it seems probable that in these experiments the conditions in which primordia isolated from the apex developed as leaves merely happened to simulate the



Text-figs. 35–40. Acropetal transverse sections of the specimen illustrated in Pl. XIII, Fig. 4 and Text-fig. 34, in which three small dorsiventral leaf primordia developed on the isolated P_1 plug. Only the P_1 plug and part of the axis are shown here in section; the shoot apex is towards the top of the page in all figures.

Text-fig. 35. At this level the vascular structure of the isolated plug is comparable with that of a single isolated leaf primordium.

Text-fig. 36. The number of discrete strands of prestelar tissue is abnormally large.

Text-figs. 37 and 38. The vascular strands now show their derivation from three separate growth centres. In Text-fig. 37 the shallow cut between P_1 and P_4 can be seen.

Text-figs. 39 and 40. The three primordia are now quite distinct; no bud is present on the plug.

Prestelar tissue black; necrosed tissue dotted. (× 15.)

effect normally exercised by the shoot apex. Primordia which develop as leaves after the surgical treatments do so in circumstances which, superficially at least, appear to be very different from the normal ones. Such primordia, indeed, still develop on the flanks of the shoot apex, but they are separated from it by a wide and deep incision, and are at a radial distance greater than usual. In some instances, when stimulated to develop by deepening the cut, the primordia were no longer above the zone of transition to the sub-apical region, but at or below it; moreover, they were sometimes surrounded on all sides by differentiating parenchymatous tissue. Growth and development of the primordia were not continuous, but were interrupted during the time that the primordia were undergoing regression, a period varying from 13 to 106 days (Table IV). As a consequence of this, the primordia did not develop in the usual definite sequence and size relationship with successive primordia. It is noteworthy, however, that notwithstanding the period of interruption in their growth the primordia usually attained a more or less normal size relationship with other primordia, doubtless as a result of their position on the sub-apical region. Indeed, they were able to regain the size of, and to surpass, primordia which, although younger in terms of plastochrone intervals, had been growing continuously for a longer time. Together with current and earlier observations on the localized transverse growth of leaf primordia as compared with the more extensive growth of buds (Cutter, 1954, 1956; Wardlaw and Cutter, 1955), this suggests that the size relationship between an apex and its incipient leaf primordia is the visible manifestation of a complex set of factors at work in the apical meristem concerned with leaf development.

This size relationship has been a source of interest to students of phyllotaxis for many years, and in a new approach to such problems Richards (1951) has given more precise expression to it in the form of a *phyllotaxis index*. This depends upon the ratio between the transverse components of the areas of the central apex and a leaf primordium at initiation, thus being expressed in morphological terms. However, Richards (1951) has also indicated that phyllotaxis index may be expressed in terms of rates of change within the apex, being then compounded of the rate of production of primordia, the rate of transverse growth of the apex in the region of the youngest primordia, and the rate of change in transverse size of the region of the apex above the zone of inception of primordia. In fact, 'Phyllotaxis index and plastochrone ratio therefore are concepts related equally to the morphology of the shoot apex and to certain rates of growth and differentiation within it' (Richards, 1951, p. 559). Under normal conditions these various processes within the apex must be very precisely synchronized; it would not be difficult to disturb them experimentally, and it is conceivable that surgical treatments might occasionally so affect metabolic processes in a region of the apex as to achieve a similar result by chance.

Previous experimental evidence suggests that a regulative effect, compounded of inhibition by growth-regulating substances, of nutrient gradients, and of competition for nutrients, is exercised upon an incipient primordium both by the shoot apex and by adjacent leaf primordia; this, indeed, is what is implied in the concept of growth fields (Schoute, 1913; Richards, 1948; Wardlaw, 1949*b*, 1952). Sussex (1952, 1953) obtained evidence of the importance of nutritional competition in maintaining the characteristic form of the shoot apex, and Allsopp (1956) has concluded that the mechanism of apical dominance cannot be attributed solely to the direct action of growth-regulating substances. The shoot apex and adjacent primordia may possibly exercise their regulative effects by controlling both the size of the growth centre and the materials available to it, for if the balance between nutrients and growth-regulating substances in the cells of a primordium is important in determining its path of development, alteration either in the quantity of substances available to the primordium or in its relative size could disturb the balance. Isolation by a wide and deep adaxial incision will alter both these conditions, resulting in the outgrowth of a larger primordium which develops as a bud. Isolation by four shallow incisions may divert the available nutrients to the wound tissue resulting from the cuts, without greatly affecting the other conditions. Subsequent deepening of the adaxial cut, however, will remove the regulative effect of the shoot apex as before, making available increased supplies of nutrients, &c., and perhaps resulting in the resuscitation of the growth centre. Conditions, however, will be different from those after initial isolation by a deep cut. For example, because of parenchymatization of neighbouring tissues the primordium may not be able to increase in size; moreover, as a result of the difference in its position and in its potential capacity for growth at the time of deepening the incision, as opposed to those of a primordium isolated by an immediate deep cut, the increase in available nutrients may be less than in the former case. In fact, the amount of nutrients might be comparable with that which would have been available to the primordium at this stage of its growth in the normal course of events. By artificially restricting the size and growth capacity of the primordium, and consequently the rates of various metabolic processes within it, these experiments may sometimes have fortuitously reproduced the regulative effects normally exercised upon the primordium by adjacent growth centres.

For a primordium to develop as a leaf, a necessary condition thus appears to be that the growth centre must be of a certain specific size and growth capacity relative to the shoot apex on which it is formed. If, in addition, there is a difference in the rates of growth in the adaxial and abaxial sides of the primordium, the organ will become of dorsiventral symmetry. However, the development of this characteristic symmetry in leaf primordia has sometimes been precluded or retarded experimentally. For example, prior to the present experiments, leaf primordia of radial symmetry have been induced by surgical means in the potato (Sussex, 1951, 1955) and also in *Epilobium hirsutum* (Snow and Snow, 1942, 1954*a*, 1954*b*), by starvation in *Osmunda* and *Onoclea* (Lang, 1924; Wardlaw, 1945), and by chemical treatments of the apex in *Dryopteris aristata* (Wardlaw, 1955*b*). In some of the present experiments the radially symmetrical primordia obtained remained so throughout the period of observation, but in others they subsequently developed as normal, dorsiventral, circinate leaf primordia. A somewhat similar condition was observed by Sussex (1955) in the course of surgical experiments on juvenile shoots of the potato, when, as a result of a certain type of incision, I_1 developed as a leaf primordium which was at first almost centric but subsequently developed a narrow lamina at a late stage in ontogeny. The present results support Sussex's conclusion that the initial outgrowth, or early development, of a leaf primordium and its dorsiventral symmetry are not necessarily conjunct.

Wardlaw (1949*a*, 1955*a*) has pointed out that the normal dorsiventrality and circinate curvature of a fern leaf primordium must result from a more rapid tangential than radial rate of growth and the early establishment of some critical difference between the adaxial and abaxial sides of a primordium. In some of the present experiments these growth relationships were affected by the surgical treatments; sometimes they became re-established in a different orientation. Thus, in some specimens in which the isolated primordia developed as leaf primordia with an initial radial phase, the deepening of the adaxial cut resulted in a considerably increased rate of growth along the radial axis of the isolated plug (Text-figs. 17–26); in some of these the primordium became dorsiventral and orientated towards one of the adjacent leaf primordia, i.e. in a plane at right angles to the normal. What decides in which of the two possible directions, i.e. in the case of P_1 towards either P_4 or P_6, it in fact becomes orientated is not clear; some critical metabolic difference between two sides of the primordium must have come into being at some subsequent stage. In this connexion, it may be noted that the vascular tissue of many of the abnormally orientated isolated primordia was found to be connected basally in the first instance with that of the adjacent primordium towards which they were orientated.

It seems worth considering if there is any correlation between unusually slow initial growth and the development of leaf primordia of radial symmetry. In many instances in which such developments have been observed the entire plants or apices were attenuated or undergoing only slow growth (Lang, 1924; Snow and Snow, 1935; Wardlaw, 1945). In *Dryopteris aristata* leaf primordia which were initially of approximately radial symmetry have previously been obtained by isolating primordia by close deep cuts on very small plugs of tissue (Wardlaw, 1947, 1949*a*; Cutter, 1956); the growth of such primordia was usually slow. Sussex (1955) states that in surgical isolations of the I_1 position in the potato shoot there was a delay in the emergence of I_1 of approximately one plastochrone. In the present experiments, also, growth of the isolated primordia was interrupted and even after stimulation by deepening the cut was usually initially rather slow.

It is of considerable interest that the three potential vegetative developments of a lateral organ on a shoot apex so far known, namely buds, radial leaves, and dorsiventral leaves—all homologous organs—have now been realized in a single species. In *Dryopteris aristata* it is thus now possible to distinguish three critical stages in the normal development of a leaf primordium; firstly its inception as a growth centre, secondly its determination as a foliar organ of limited growth, and thirdly its development as an organ of dorsiventral symmetry. Under normal conditions the second and third stages must be in relatively close temporal relationship, since leaf primordia of radial symmetry are never observed. The development of a radial leaf primordium might therefore be considered as an abnormal prolongation, as a result of unusually slow growth, of the second phase of development, sometimes resulting in the total prevention of the onset of the third phase. In other species, e.g. in many flowering plants, two or more of these processes may be so nearly contemporaneous as to be inseparable by existing experimental means.

SUMMARY

1. On inactive apices of *Dryopteris aristata* young visible leaf primordia not already determined as foliar organs, and a few presumptive leaf sites, were isolated by four shallow incisions, resulting in the regression and disappearance of the primordia; one or more of the shallow incisions was subsequently deepened, usually when the primordia were just at the point of disappearance.

2. When the adaxial incision was subsequently deepened, the growth centres were sometimes stimulated into renewed growth; the majority of these primordia developed as buds, but about a quarter of those which reacted to the stimulus developed as leaves.

3. A greater proportion of primordia failed to respond to the stimulus of deepening the adaxial cut when the initial shallow incisions were made close to the growth centre.

4. Subsequent deepening of the abaxial incision had no stimulatory effect in the small number of specimens observed; in the majority of cases subsequent deepening of both radial incisions was also without effect, but in one specimen a laterally orientated leaf primordium developed.

5. No correlation could be established between the development of an undetermined isolated primordium as a leaf and (*a*) its stage of development at the time of the initial isolation by shallow incisions, (*b*) its external appearance at the time of deepening the adaxial incision, (*c*) the time which elapsed between the initial isolation and deepening the cut, or (*d*) the rate of growth of the apex during this time.

6. Some primordia which developed as leaves after subsequent deepening of the adaxial incision were of normal dorsiventral symmetry; some were radially symmetrical, and others underwent a phase of radial development before becoming dorsiventral.

7. Some isolated primordia which developed as dorsiventral leaf primordia were normally orientated towards the shoot apex, others were orientated laterally or somewhat obliquely towards one of the adjacent leaf primordia. Lateral or axillary meristems were sometimes present on the isolated plugs.

8. For leaf determination to take place, it is not sufficient for a piece of

tissue to remain in contact with the shoot apex for a certain length of time; a primordium is only competent to be determined at a certain stage of its growth.

9. It seems likely that in specimens in which the isolated primordia developed as leaves, the experimental treatments have fortuitously reproduced the effects normally exercised by the shoot apex, or the growth relationships normally obtaining.

10. It is suggested that one of the necessary conditions for the development of a primordium as a leaf may be the maintenance of a specific relationship between its size and growth capacity and those of the shoot apex on which it is formed, and that the regulative effects of the shoot apex and adjacent primordia may function by creating and maintaining this relationship.

11. The more rapid tangential growth and the metabolic differences between the adaxial and abaxial faces of a primordium which, as Wardlaw (1949*a*, 1955*a*) has pointed out, must bring about the normal dorsiventral symmetry and circinate curvature of a fern leaf were usually altered by the experimental treatments, and in some cases re-established in a different orientation. Deepening of the adaxial cut often resulted in an enhanced rate of radial growth. Orientation may conceivably have been influenced by the establishment of the first vascular connexions; this might also explain its tardy manifestation.

12. On the basis of these and earlier observations, three phases in the development of a leaf primordium of *D. aristata* can now be differentiated: (i) the inception of a growth centre, (ii) its determination as an organ of limited growth, and (iii) its development as an organ of dorsiventral symmetry.

13. It is suggested that under normal conditions of growth in *D. aristata* phases (ii) and (iii) must be in relatively close temporal relationship, but that under conditions of slow growth a phase of radial symmetry may become manifest in the development of a leaf primordium, at least in the configuration of its vascular system.

14. In other species the temporal relationships between these three phases may differ, so that two or more may be inseparable by comparable experimental treatments.

ACKNOWLEDGEMENTS

I should like to express my thanks to Professor C. W. Wardlaw for much helpful discussion and advice during the preparation of this paper, and to Mr. E. Ashby for the photographic illustrations.

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EXPLANATION OF PLATES

Illustrating E. G. Cutter's paper on 'Further Experiments on the Developmental Potentialities of Leaf Primordia in *Dryopteris aristata* Druce'.

PLATE XII

Fig. 1. Surface view of a specimen in which P_1 was isolated by four shallow cuts and the adaxial cut was deepened 35 days later, after P_1 had almost disappeared and was flatter than I_1 and I_2. Twenty-six days after deepening the cut, as shown here, P_1 had developed as a

normally orientated dorsiventral leaf primordium, possibly slightly smaller than normal. Phyllotaxis clockwise, from younger to older primordia. I_1–I_6 present. Compare Text-fig. 14. (×40.)

Fig. 2. Surface view of a specimen in which the I_2 presumptive position was isolated by four shallow cuts and the adaxial incision was deepened 22 days later. I_2 developed as a normally orientated, dorsiventral, circinate leaf primordium of approximately normal size, and is shown here 69 days after deepening the adaxial incision, with P_2 on the left and P_4 on the right. *r-r'*=shallow radial incisions; *ai*=deepened adaxial incision. The position of the shoot apex is towards the foot of the page. Compare Text-fig. 16. (×40.)

PLATE XIII

Fig. 3. Surface view of a specimen in which P_1 was isolated by four shallow cuts and the adaxial incision was deepened 35 days later, when P_1 was still just visible but quite flat. Twenty-two days after deepening the cut, as shown here, P_1 had developed as a leaf primordium of normal size, apparently of radial symmetry. Its position was outlined by incipient scales. Subsequently a small bud developed in a lateral position. Phyllotaxis clockwise; I_1–I_4 have been formed. Compare Text-fig. 27; see also Pl. , Figs. 8 and 9. (×40.)

Fig. 4. Surface view of a specimen in which P_1 was isolated by four shallow cuts and the adaxial incision was deepened 24 days later, when P_1 was still just visible and a few incipient scales were arising at the periphery of the rather concave plug. At first little growth took place, but 6 weeks after deepening the cut, as shown here, three small dorsiventral leaf primordia, orientated towards a central scaly hollow, were present on the plug. The isolated plug is shown here, with P_6 on the right and the damaged P_4 on the left; the shoot apex is towards the bottom of the page. *r-r'*=shallow radial incisions; *ai*=deepened adaxial incision. Compare Text-fig. 34; see also Text-figs. 35–40. (×40.)

PLATE XIV

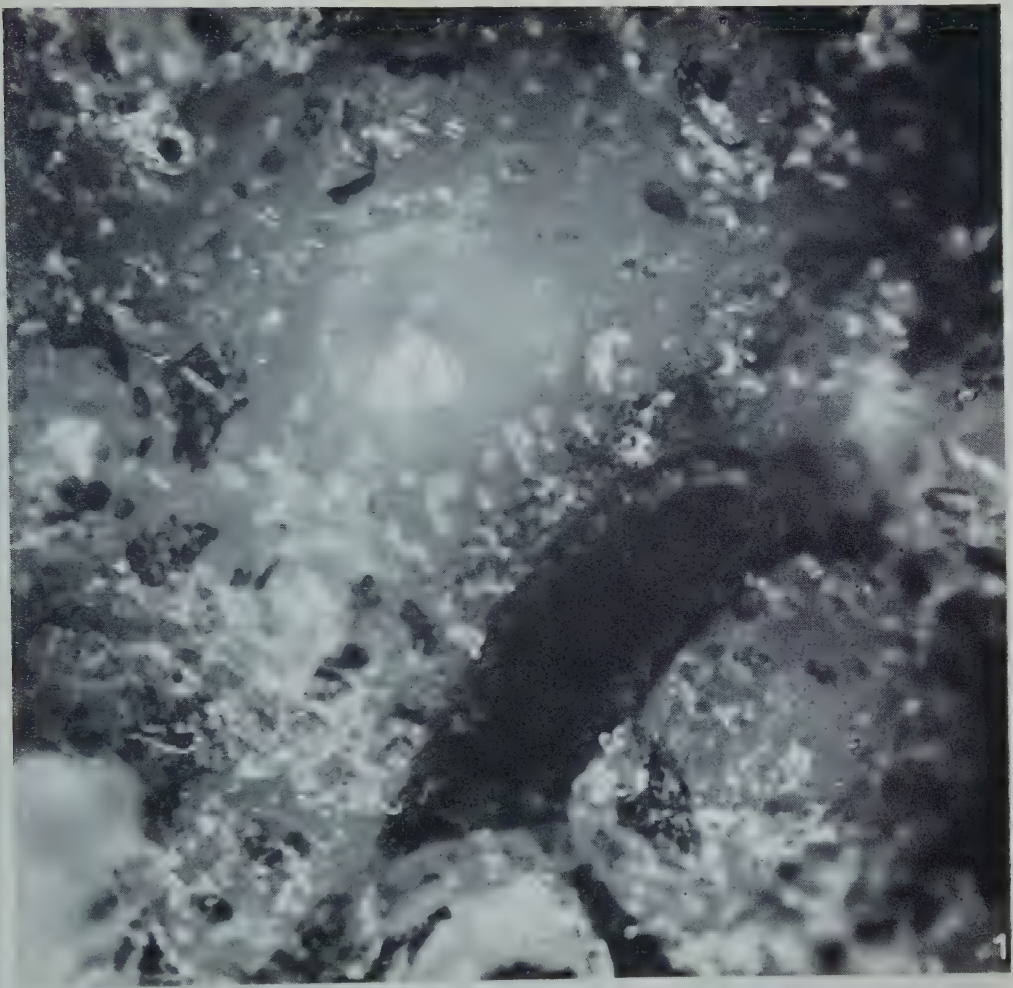
In all figures the position of the shoot apex is towards the top of the page.

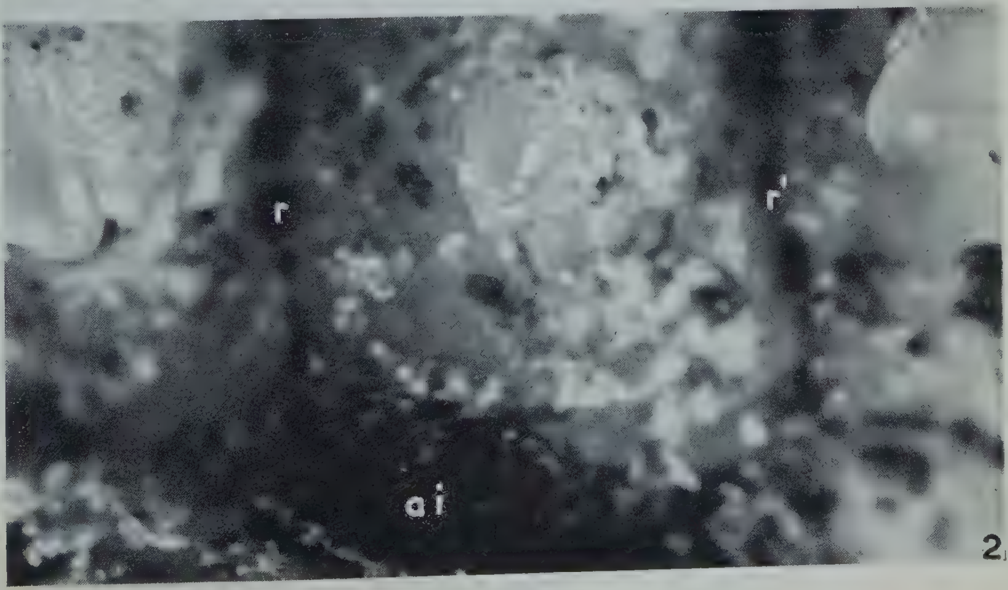
Fig. 5. Transverse section of an apex in which the I_1 presumptive position was isolated by four shallow incisions with subsequent deepening of the adaxial cut. I_1 developed as a double leaf primordium of dorsiventral symmetry, orientated towards the abaxial P_3 corner of the plug. This section, near the junction of the isolated plug with the axis, shows the solenostelic nature of the vascular tissue of I_1 at this level. At higher levels a ring of discrete vascular strands was present. (×25.)

Fig. 6. Transverse section of a leaf primordium which developed from a P_1 primordium isolated by four shallow cuts with subsequent deepening of the adaxial incision. The leaf primordium became dorsiventral and circinate, and during external observations was not judged to be certainly of radial symmetry at any time. The sections, however, show the radially symmetrical arrangement of the prestelar tissue in the form of a solenostele. (×70.)

Fig. 7. Transverse section of an isolated P_1 primordium which developed as a dorsiventral, pinnate leaf primordium with a small bud lateral to it. The leaf passed through a phase of radial symmetry which was observable externally and is here shown in section. (×35.)

Figs. 8 and 9. Acropetal transverse sections of the specimen illustrated in Pl. , Fig. 3 and Text-figs. 27–28, in which an isolated P_1 primordium developed as a leaf primordium of radial symmetry. A small bud was later formed in a lateral axillary position on the isolated plug. The vascular systems of the radial leaf and of the bud can be seen; the radially symmetrical ring of strands of prestelar tissue in the leaf is well seen. (×35.)





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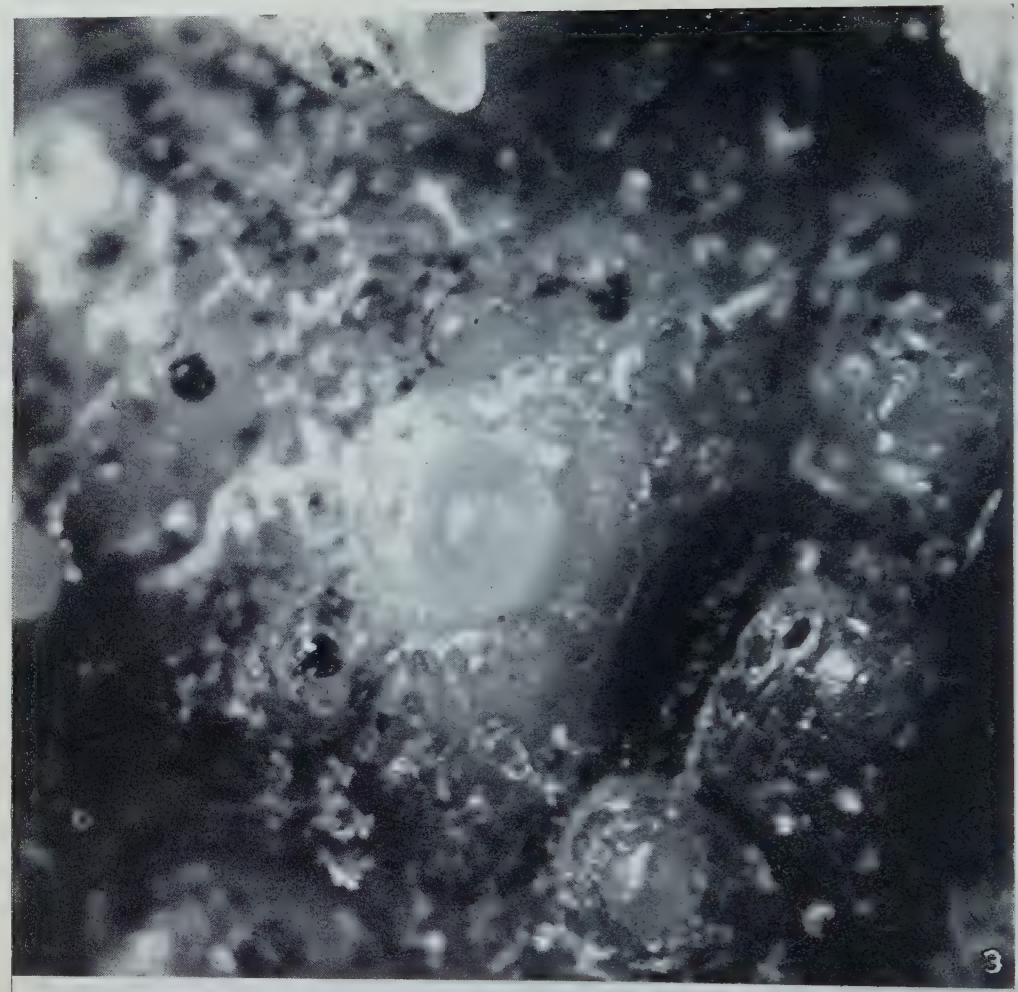

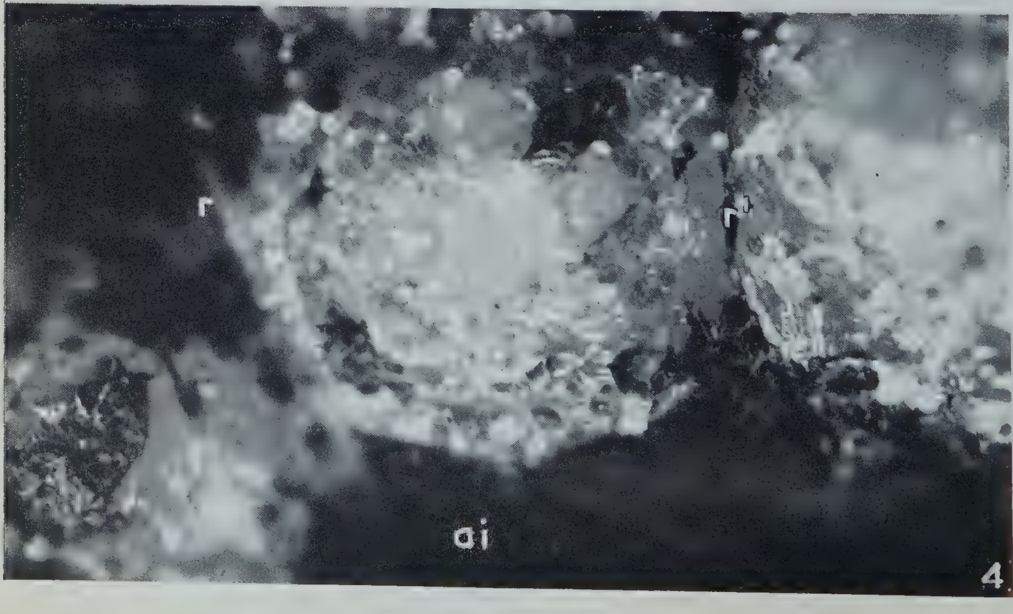

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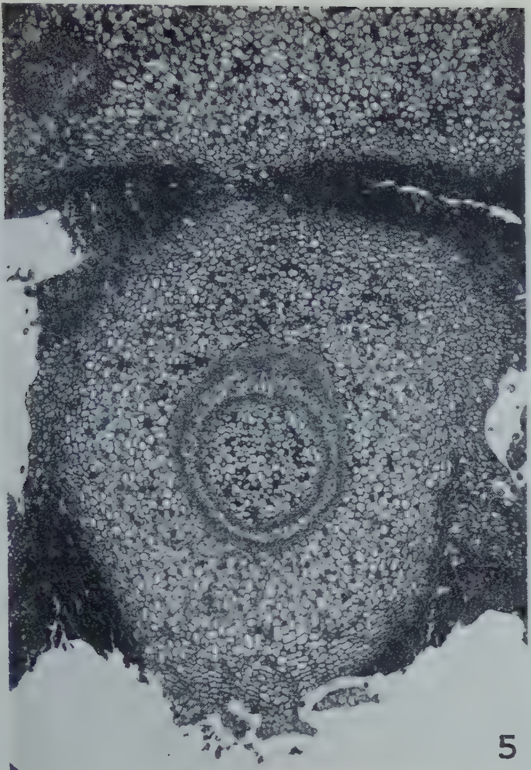

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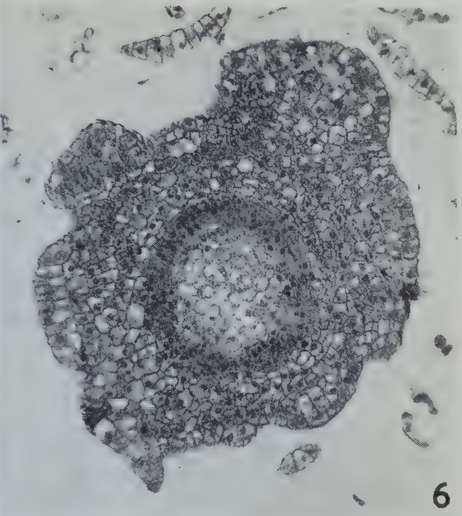

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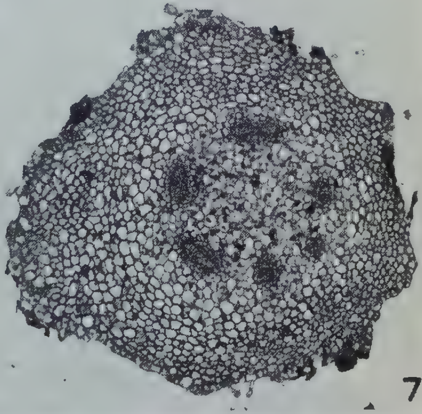

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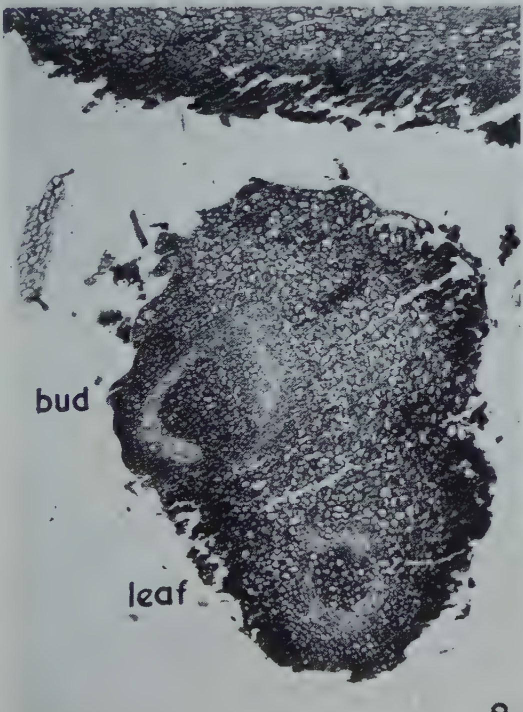

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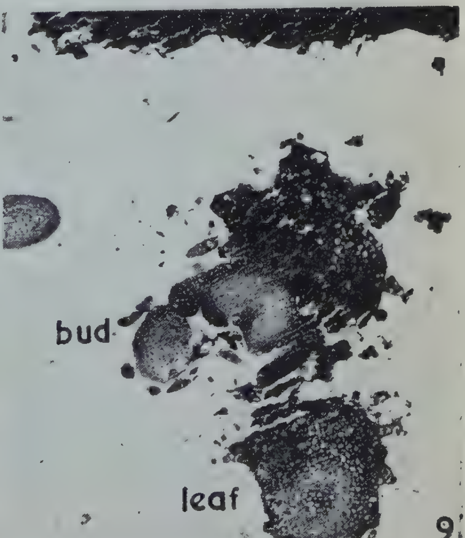

9

E. G. CUTTER

The Development and Significance of the Root Nodules of *Casuarina*

BY

G. BOND

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With Plate XV and one Figure in the Text

Abstract

Young plants of *Casuarina cunninghamiana* and of *C. equisetifolia* growing in water culture developed macroscopic nodules in 26 to 35 days from inoculation, the nodulation being most successful at pH near to neutrality and falling off much more rapidly at lower pH than in other non-legume nodule-forming genera, confirming the distinctiveness of the *Casuarina* organism. The roots springing from the nodule lobes are shown to be characterized by upward growth and in this to resemble remarkably closely the corresponding roots of *Myrica*. Nodulated plants of *Casuarina* are able to grow vigorously in culture solution free of combined nitrogen, showing that fixation of atmospheric nitrogen occurs, amounting in *C. cunninghamiana* to 50 mg. per plant during 6 months of active growth. The evidence indicates that the fixation occurs in the nodules, and that these have exactly the same functional significance as those of legumes.

Introduction

THE genus *Casuarina* is the most important economically of the small group of non-legume Angiospermous genera that bear root nodules, and the development and physiological significance of the nodules of the genus deserve more attention than they have hitherto received. The majority of previous investigators of *Casuarina* nodules have concerned themselves with the identity of the endophyte, several having isolated bacterial organisms from the nodules, without, however, establishing by subsequent reinoculation that the true endophyte had been obtained. References to these investigations may be found in papers by Aldrich-Blake and by Mowry (see below). Schaede (1948), on the basis of cytological studies of the nodule, classed the endophyte as an Actinomycete, as he did the endophytes of all the other non-legume genera.

The significance of the nodules in the nitrogen-nutrition of the *Casuarina* plant was first investigated in little-known work by Aldrich-Blake (1932) and by Mowry (1933). They carried out what may be termed experiments of the Hellriegel and Wilfarth type, in which the growth of nodulated and non-nodulated plants in pot culture in sand low in combined nitrogen was compared. The experiments of the former author were carried out at Dehra Dun, those of Mowry in Florida. Both found that the nodulated plant grew vigorously under the conditions indicated. Thus Aldrich-Blake found that after 14 months' growth, starting from seed, nodulated plants of *C. equisetifolia*

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showed a mean nitrogen content of 600 mg. per plant, compared with 17 mg. for non-nodulated plants, with proportionate differences in size. Mowry obtained similar results with *C. equisetifolia*, *C. cunninghamiana*, *C. glauca*, and *C. lepidophloia*. These findings indicate that fixation of atmospheric nitrogen is associated with nodulated *Casuarina* plants.

In the present work confirmation and extension of the above results have been sought using the alternative technique of water culture which permits of closer study of nodule development and the effect on it of various factors than is possible in sand culture.

METHODS

Seed of *C. cunninghamiana* and *C. equisetifolia* was germinated without surface-sterilization in April and May 1955 in trays of moist sand. When the epicotyledonary shoot had attained a height of about 5 mm. the young plants were transplanted into water culture, 2-litre glazed earthenware jars being employed, covered by teak tops in which usually 7 plants were supported. In most experiments the jars contained Crone's solution made up to a nitrogen-free formula with added minor elements as used by Bond (1951), or a diluted form of this. At this early stage 20 mg. combined nitrogen as ammonium sulphate was added per litre of culture solution and the plants remained in this solution until shortly before inoculation.

With the assistance of Dr. H. R. Fletcher of the Royal Botanic Garden, Edinburgh, and the kind co-operation of the directors of botanic gardens abroad, living nodule material was received by air mail as follows: of *C. sumatrana* from Singapore, of *C. equisetifolia* from Bogor-Djawa, of *C. cunninghamiana* from Melbourne, and of *C. glauca* from Sydney. Nodules of the second species were also received from the Sub-Tropical Experiment Station, University of Florida. These samples of nodules were used in the preparation of inocula for the experimental plants. Observations made by Mowry (1933) indicate that the organisms from nodules of different species of *Casuarina* are cross-inoculable if not in fact identical, so that it is immaterial, at least from the point of view of securing nodule formation, if the nodules to be used for inoculation are from another species of *Casuarina*. On the day prior to inoculation the plants were transferred to culture solution free of combined nitrogen, in some experiments the pH of the Crone's solution, which without adjustment is 6·4, being modified to desired levels by the addition of acid or alkali. Inocula were prepared by grinding the nodules in water, the weight of nodules per 100 ml. water varying from 8 to 30 gm. in different experiments. The preparation so obtained was filtered through muslin and then brushed on to the roots of the plants to be inoculated. Inoculation was carried out at different dates in the period July to September, 1955. The height of the shoots of the plants at the time of inoculation varied from 5 to 8 cm. in different experiments.

Uninoculated plants were also grown, some in nitrogen-free solution as controls, others supplied with ammonium-nitrogen. The culture solution was renewed at intervals of 3 to 4 weeks; where it was desired to keep the pH of the solution as constant as possible the value was checked every few days and readjusted as necessary. The cultures were kept in a greenhouse with daytime temperature of 18° to 30° C. in summer and 10° to 15° C. in winter.

DATA OBTAINED

Time required for nodule development

In one experiment macroscopic nodules were first present on the plants 26 days after inoculation, this being the shortest interval experienced. In three other experiments the interval was about 35 days. The only previous record of this type is in the paper by Mowry (1933), the interval there being much longer, due, no doubt, to a less efficient method of inoculation.

Features presented by young nodules

Examination of roots showed that an early effect of inoculation was a pronounced deformation and branching in the root hairs, which in uninoculated plants were straight and unbranched. This effect was very reminiscent of that shown in inoculated legume roots. Further study of the infection process has not yet been made.

The nodules commence as swellings on lateral roots, and under the conditions of the experiments these nodules were often of intense red colour, a feature found also in *Alnus* and *Myrica* nodules and shown by Bond (1951) in the last case to be due to anthocyanin pigments. As a result of branching the young nodule develops a lobed structure, and as noted by McLuckie (1923) and other authors a root soon grows from the tip of each lobe, representing a continuation of the root of which the swollen basal region forms the nodule lobe. It soon became obvious that these roots are characterized by upward growth, a finding which was reported, so far as is known for the first time, in a preliminary note (Bond, 1956*a*). This feature is illustrated in Pl. XV, Figs. 1 and 2. In older plants these nodule-roots form a very conspicuous feature of the root system.

Effect of pH on nodule initiation

Fig. 1 indicates that a pH near to neutrality is most favourable to infection of the roots. The plants themselves at pH 4·0 showed no sign of acid-damage, and it may be concluded that the adverse effect was exerted on the nodule organism.

The subsequent growth of nodulated plants

Evidence of the commencement of fixation of atmospheric nitrogen was provided by the gradual resumption of growth of the plants after nodules had appeared, despite the lack of combined nitrogen in the rooting medium; the control plants, uninoculated and remaining without nodules, developed increasing signs of nitrogen starvation. After the winter period the nodulated plants resumed vigorous growth. In the various experiments some 200 nodulated plants of *C. cunninghamiana* were grown, and all developed strongly apart from those which because of unfavourable pH or for other reason formed only few nodules. A detailed study was made of 12 nodulated plants of this species, representative of the 50 plants of a particular experiment, the plants being harvested when they had experienced a total of 6 months of reasonably favourable weather subsequent to nodulation. A photograph of typical nodulated and control plants is provided in Pl. XV, Fig. 3, and the harvest data are presented in Table I. The superiority of the nodulated plants over the control plants is very obvious. Since the mean nitrogen content of the nodulated plants is 51 mg. and that of the control plants approximately 1 mg., it may be concluded that a mean fixation of 50 mg. per plant occurred during the stated period. Mean percentage nitrogen (dry-weight basis) was 2·95 in the nodules and 2·36 in the rest of the plant.

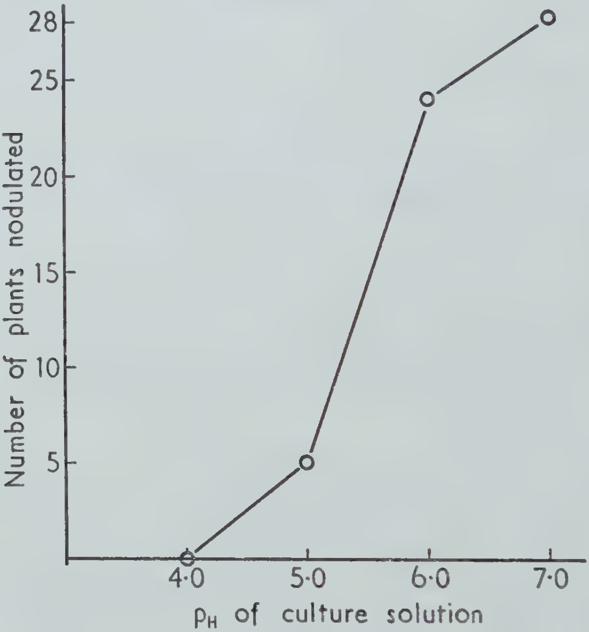

FIG. 1. Effect of pH of culture solution on nodule development in *C. cunninghamiana*. There were 28 plants at each pH, the inspection for nodules being carried out 10 weeks after inoculation.

Six nodulated plants of *C. equisetifolia* grown for the same period as the previous species were similarly examined, together with control plants. Due, as was realized subsequently, to the rather low pH (5·4) at which these plants were grown, the proportion of inadequately nodulated plants was rather high and growth was more variable. The mean nitrogen content of the plants analysed was 25 mg. per plant, compared with 0·7 mg. for the control plants.

Percentage nitrogen in the nodules was 2·81, and in the root and shoot together 1·68. Typical plants are shown in Pl. XV, Fig. 4.

Other observations made were that *Casuarina* grows more freely if the Crone's solution is used at half its normal strength, and that the few plants which succeeded in forming a good number of nodules at pH 5 subsequently grew quite strongly at that rather low pH.

TABLE I

Harvest Data for Plants of Casuarina cunninghamiana*

Type	Plant number	Height of main shoot (cm.)	Dry weight (g.)		Total nitrogen (mg.)		
			Nodules	Root and shoot	Nodules	Root and shoot	Whole plant
Nodulated	1	41	0·140	1·60	4·0	40·9	44·9
	2	42	0·095	1·43	2·9	36·9	39·8
	3	49	0·152	2·70	4·4	63·7	68·1
	4	47	0·157	2·25	4·8	50·7	55·5
	5	39	0·077	1·20	2·3	33·5	35·8
	6	38	0·084	1·43	2·6	38·1	40·7
	7	62	0·169	2·85	4·9	65·3	70·2
	8	44	0·142	2·42	4·1	54·9	59·0
	9	38	0·085	1·11	2·5	30·4	32·9
	10	46	0·139	2·31	4·1	56·6	60·7
	11	44	0·113	1·91	3·2	37·3	40·5
	12	47	0·175	2·72	5·3	58·0	63·3
Non-nodulated controls	1 to 6	Range 11 to 18	—	Range 0·07 to 0·17	—	Range 0·6 to 1·5	

* Seedlings were transplanted from sand tray into water culture with combined nitrogen on July 4, 1955. Plants transferred to nitrogen-free solution July 21 and inoculated (except for control plants) on the following day with inoculum prepared from *C. equisetifolia* nodules from Bogor-Djawa; harvested July 18, 1956. For most of the period the plants were in half-strength Crone's solution pH 6·4, falling to pH 4·5 by the time the solution was renewed.

DISCUSSION

The data presented above confirm that fixation of atmospheric nitrogen is associated with nodulated *Casuarina* plants. The experiments, in common with those of the two previous authors mentioned in the Introduction, were not of aseptic type, and it cannot be claimed that the nodule endophyte was the only micro-organism present in the rooting medium. But the circumstances indicate very definitely that the fixation is a nodular function. It is only shown by nodulated plants, and its extent runs parallel to the development of the nodules; if the nodules are removed from a plant symptoms of nitrogen deficiency soon arise. No fixation of nitrogen is associated with plants that have failed to form nodules, even though they are in the same jars as vigorously fixing, nodulated plants. Thus there is every evidence that as already demonstrated by Bond, Fletcher, and Ferguson (1954), Quispel (1954), and Bond, MacConnell, and McCallum (1956) for *Alnus*, *Myrica*, and *Hippophaë*, so in *Casuarina* the nodules resemble closely those of legumes in their significance. The nodulated plant stands in the same relation to nitrogen as does the nodulated legume.

There is no reason to doubt that the fixation demonstrated in cultured plants will also be shown in the field, provided the plants are nodulated. In *Alnus* Bond (1956*b*) demonstrated by direct test using isotopic nitrogen that the fixation shown in greenhouse cultures is also characteristic of nodules of field plants. The literature indicates that nodulation in *Casuarina* is common in regions to which the genus is indigenous, and also that it occurs in some other areas. As examples of the latter, Mowry (1933) found nodulation widespread in Florida, and also had evidence of its occurrence in Dominica, Honduras, and the West Indies. Dr. J. H. Palmer, University College of the West Indies, has informed the present writer of the abundant nodulation of *Casuarina* in Jamaica. Possibly the organism is of wide distribution in tropical soils, existing in a non-symbiotic condition in the absence of the normal host plant. The organism appears to be absent from the soils of more temperate regions, since Miehe (1918) reported a lack of nodules on *Casuarina* plants in German botanic gardens and also in Italy and Egypt, and the present writer has been unable to learn of any nodules in British botanic gardens.

As pointed out by Aldrich-Blake (1932), the natural pioneering of sandy coastal areas by *Casuarina* species, especially *C. equisetifolia*, is in keeping with the possession of special facilities for gaining nitrogen. It cannot be doubted that the effect is eventually to raise the fertility of the soil. Thus this widely distributed genus should be accorded special significance as a source of combined nitrogen for other plants.

Certain values derived from the data reported above are presented in Table II along with comparable values for other nodule-bearing plants. All are based on experiments conducted in the one greenhouse under generally similar conditions, those for the perennial non-legumes referring to the first year of development. The entry in the left-hand column of figures provides an index of the efficiency of the nodule tissues. The value for *Casuarina* is below most of the others, but this is possibly because the climatic conditions of the experiment were less nearly optimal for this than for the majority of the plant species. In the right-hand column the general similarity of the value for *Casuarina* to those for legumes suggests that similar correlative influences operate during development, and is in keeping with the other evidence of a close physiological comparability between the nodules. The symbiosis in *Casuarina*, from the standpoint of the higher plant, did not under the prevailing conditions attain the high efficiency of that of *Alnus*, where organs accounting for only 2·2 per cent. of the total dry weight provided sufficient fixed nitrogen to support very vigorous growth of the host plant.

It is of interest that the roots which spring from the nodules in *Casuarina* should prove to show the same upward growth previously demonstrated in the nodule-roots of *Myrica* (Bond, 1951, 1952), where the effect was observed in various rooting media and under field conditions. It should be mentioned that the nodule-roots in the nodule material received from abroad in connexion with the present work were in a shrivelled state on arrival, so that their original orientation could not be determined. As in *Myrica* the tendency to upward growth is not confined to the nodule-roots in *Casuarina*, since higher orders of ordinary roots sometimes show the same feature in cultured plants, though less strikingly. McLuckie (1923) envisaged the ventilation of the *Casuarina* nodule as occurring by means of imperfections in the cork covering or through the unsuberized tip. It is possible that the subsequent development of the upward-growing nodule-root assists in nodule ventilation.

In failing to infect the roots at pH 4, and in infecting them only poorly at pH 5, the *Casuarina* organism differs sharply from those of *Alnus* and *Myrica*, which have a much greater acid-tolerance, and to a lesser extent from that of *Hippophaë* (Bond, Fletcher, and Ferguson, 1954). This bears out that the *Casuarina* organism is not identical with any of the above, as is also indicated by the failure of *Casuarina* to nodulate in European soils.

TABLE II

Comparative Data for Casuarina *and other Nodule-Bearing Plants**

	Mg. N fixed per g. nodule dry matter formed	Nodule dry wt. as % of whole plant dry wt.
Field bean (*Vicia*)	691	4·0
Soya bean	258	8·9
Soya bean†	66	5·2
Field pea	539	5·3
Red clover	703	4·0
Alnus	814	2·2
Myrica	420	6·6
Hippophaë	741	3·6
Casuarina	391	6·1

* Certain of the data in the table are reproduced from Ferguson and Bond (1953). The data for *Casuarina* refer to *C. cunninghamiana*.

† With ineffective strain of rhizobium.

Finally it may be noted that Bose (1947) referred to his isolation of a fungus, designated *Phomopsis casuarinae*, from seeds and other parts of *Casuarina* plants; presumably it is to this organism that Wilson (1952) refers, under the name *Phoma casuarinae*, stating that the organism had been sent by Bose to Madison for test by the isotopic method for nitrogen-fixing power, for which there was slight cultural evidence. Difficulties had been encountered in the tests, but on the whole it seemed that there was no fixation. In any case it will be best to regard this mycorrhizal association of *Casuarina* as something apart from the association with the nodule endophyte. It is the latter to which the present paper refers; it may be relevant, however, to stress that plants raised from unsterilized seed showed no ability to fix nitrogen until they had become nodulated.

ACKNOWLEDGEMENTS

In addition to indebtedness already expressed, the author is grateful to Sir Edward Salisbury for advice on the procurement of *Casuarina* seed. The photography was by Mr. W. Anderson and Mr. R. Cowper, and technical assistance was given by Miss K. McCormick.

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EXPLANATION OF PLATE XV

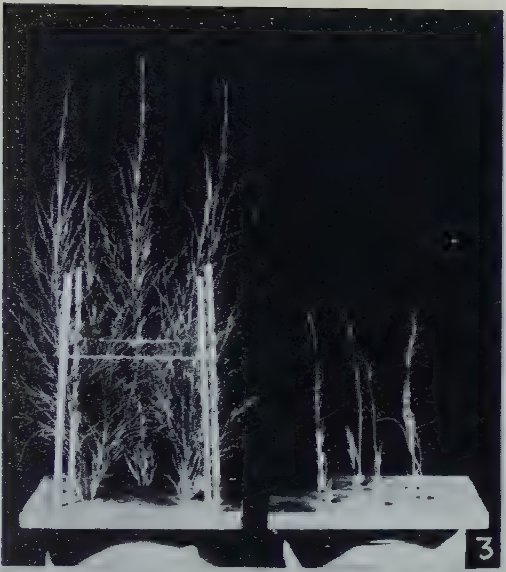
Illustrating the article by G. Bond on 'The Development and Significance of the Root Nodules of *Casuarina*.'

Fig. 1. Root system of plant of *C. cunninghamiana* after 3 months' growth, showing the upward-growing roots springing from the nodules. (×½.)

Fig. 2. Detached parts of nodule clusters, with nodule roots, photographed in their normal orientation. (×2.)

Fig. 3. Nodulated (left) and non-nodulated control plants of *C. cunninghamiana* after 6 months' growth in solution free of combined nitrogen. (×⅙.)

Fig. 4. As Fig. 3 except that here the plants are of *C. equisetifolia*. (×⅙.)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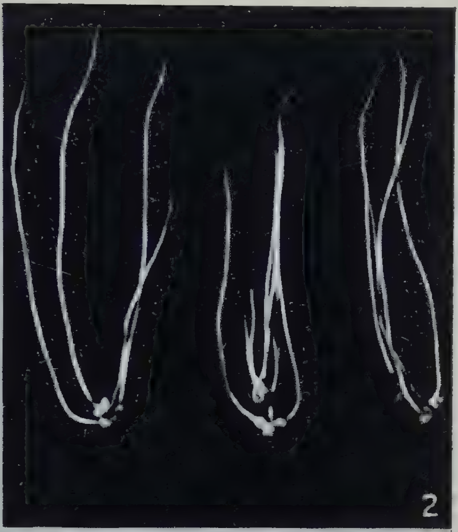
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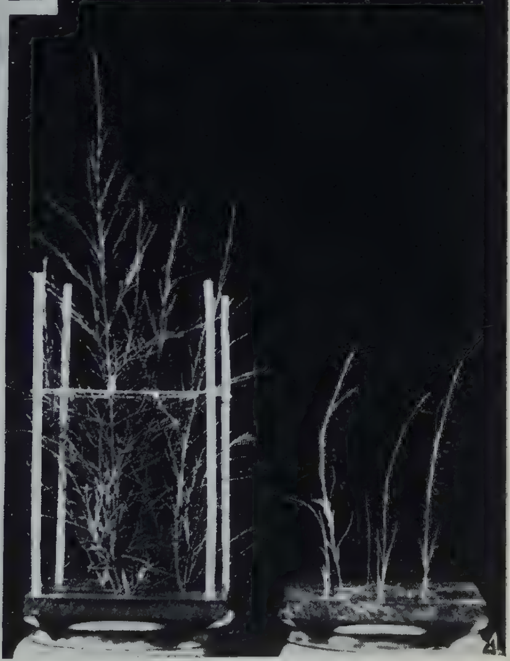
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G. BOND

Studies on the Absence of Junctura, Auricle, and Ligule in Rice, *Oryza sativa* L.[1]

BY

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With one figure

ABSTRACT

A case of complete absence of junctura, auricle, and ligule in rice is reported. Absence of the characters is found to be dominant to presence. All the three characters seem to be linked.

The material reported is expected to be useful in establishing the genetic relationship between the rudimentary auricled condition and the absence of auricle.

The majority of the improved rice varieties evolved by the Agriculture Departments of the different States of India are the result of pure line selection. As such, the improved variety is morphologically indistinguishable from the unimproved bulk. It is, therefore, necessary to evolve a method by which the improved variety can be easily distinguished (in the field as well as in the godown) from the unimproved bulk.

The absence of the junctura, auricle, and ligule may, with advantage, be used as a 'Marker' for tagging the improved varieties.

INTRODUCTION

CONSIDERABLE progress in the genic analysis of rice, *Oryza sativa* L., has been made. Nearly 300 genes covering about 50 major characters are recorded. Kadam and Ramiah (1943) proposed gene symbols and indicated their use. Among the characters studied, mention may be made of junctura, auricle, and ligule. The present paper deals with a case wherein junctura, auricle, and ligule are all absent.

Junctura. A juncturaless condition was not reported by Kadam and Ramiah (1943).

Auricle. Kadam and Ramiah (ibid. 1943) recognized the factor pair governing the presence and absence of auricle and termed it Au-au.

Hector (1936) reported an auricleless condition in rice. Chiapelli (1936) also described a mutant named Balzaretti which is completely auricleless. Piacco (1939) referred to a case of a mutant lacking auricles (and liguleless). The auricleless condition was recessive and was controlled by one factor pair. Ghose and Butany (1952) stated that in the world collection of nearly 3,000 rice genetic stocks available at the Central Rice Research Institute, Cuttack, they did not come across any type without auricles. However, they report

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[1] Based on a paper read at the 41st Session of the Indian Science Congress, Hyderabad, 1954.

[Annals of Botany, N.S. Vol. xxi, No. 83, 1957.]

cases wherein the auricle is rudimentary. They also state that a single factor pair determines the fully auricled (dominant) and rudimentary auricled condition (recessive). It is further stated that the relationship between the rudimentary auricle and the auricleless condition has yet to be determined.

Ligule. An absence of ligules is more frequent (Kadam and Ramiah, 1943) and Lg-lg are the recommended symbols for the factor pair.

Jones (1933), Morinaga (1938), and Piacco (1939) report a monogenic ratio for the presence and absence of ligule, the absence being recessive.

MATERIAL AND METHODS

In Abi[1] 1952-3, Shala, a variety locally grown in Kashmir State, was grown for the first time at the Main Agricultural Experiment Station, Himayet Sagar, Hyderabad. The population showed two types of plants. In one, the plants showed junctura, auricle, and ligule, all three characters fully developed. In the other type, all these three characters were completely absent. The complete absence of junctura is specially noteworthy as this is the first time that such a condition is reported on. The differences between the two types of plants is shown in the accompanying illustration.

In the juncturaless, auricleless, and liguleless type, the lamina looks a mere extension of the sheath with a very slight constriction at the point that should have been the junctura. The usual angle formed by the lamina and the leaf sheath at the junctura is absent and the lamina runs more or less parallel to the stem.

In January–May 1952–3, 119 single plants were studied individually and harvested. Out of these, 104 plants showed complete absence of junctura, auricle, and ligule while only 15 plants showed the presence of all three characters. In the succeeding season the progenies of these 119 individual plants were studied separately. Out of the 104 strains that showed absence of junctura, auricle, and ligule, 5 failed to germinate. Thirty-one out of the remaining 99 bred true. The rest segregated into two types, one not having any of the three characters and the other having all the three characters, i.e. junctura, auricle, and ligule. No type possessing any two of the characters was observed. Nor was the intermediate rudimentary condition noted. The segregates in the progeny of the juncturaless, auricleless, and liguleless showed that out of a total of 1,723 plants, 1,539 lacked all three characters while 183 showed junctura, auricle, and ligule. This shows that the absence of the three characters behaved as a single unit and is dominant to the presence.

The progeny of the fifteen selections amounting to 972 individual plants, bearing junctura, auricle, and ligule bred true.

DISCUSSION

The presence or absence of junctura, auricle, and ligule reported in this paper may prove to be of theoretical interest and of practical utility.

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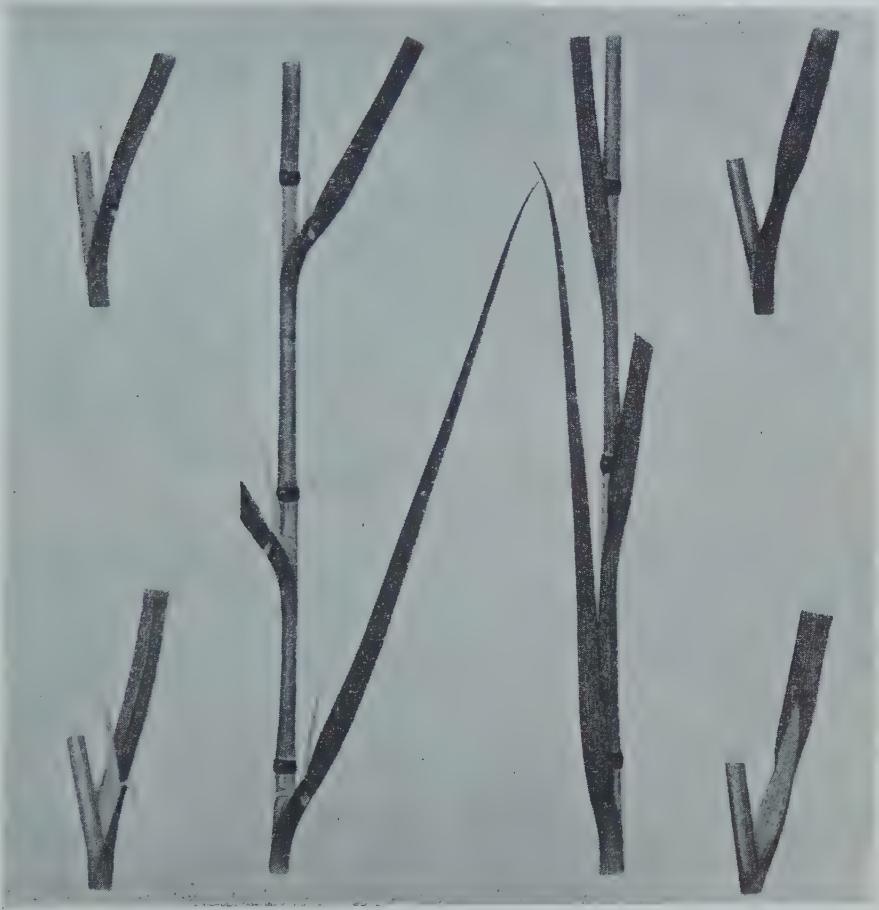
[1] The rice season in Hyderabad extending from June to December.

The seed of local Shala, originally received from Kashmir, evidently contained some natural hybrids. It is hard to state at the present stage whether the seed belonged to F_2, F_3, F_4 . . . generation. But the fact that it was segregating right from the start establishes its hybrid nature beyond F_1. The genic analysis of the characters is not possible unless some more generations are thoroughly studied. This is in progress, but it can be said with fair confidence that the absence of junctura, auricle, and ligule is dominant to their presence. This observation is at variance with that reported by earlier workers who showed that presence is completely dominant to absence.



Normal Shala showing presence of junctura, auricle, and ligule

Natural hybrid of Shala showing absence of junctura, auricle, and ligule

Exceptional variations in junctura, auricle, and ligule.

The present material is expected to be helpful in mapping the chromosomes of rice. It may also help in establishing the relationship between rudimentary auricles and the auricleless condition.

While junctura, auricle, and ligule are not of any economic value, the absence of these characters, occurring together, may be made of use as a 'Marker' in breeding projects. The continued maintenance of genetic purity in the improved varieties of rice is an absolute necessity in every state or for that matter in every country. Ramiah (1951) has stated that the total number of improved rice strains under distribution in India is 284. Out of these, only 27 are of hybrid origin. In other words, the bulk of the improved strains that contribute towards increased production are a result of pure line selection. Since pure line selection is based mostly on yield performance, it is not possible to distinguish morphologically the selected strain from the originals. To illustrate, HR. 35, the second most popular improved variety of Hyderabad State, is a selection from the local 'Kichdi Sannal' and the two are indistinguishable in the field as well as after harvest. Therefore, a triple cross synthesizing (*a*) the favourable features of HR. 35, (*b*) the absence of junctura, auricle, and ligule of Shala, and (*c*) the long outer glume character of *Neeladharulu*, a local variety, will enable workers to identify the improved variety in the field as well as in the godown.

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Observations on the Occurrence of Prickles on the Leaves of *Ilex aquifolium*

BY

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(*University of Manchester*)

With thirteen Figures in the Text

ABSTRACT

The number of prickles upon the edge of a leaf is related by complex laws to the size of the leaf, the number of prickles on the other edge, the number of prickles on other leaves of the shoot, the phyllotaxy of the shoot, and the intrinsic asymmetry of the leaf. The available evidence indicates that prickles arise by the interaction of not less than five recognizable physiological systems. The physical nature of these systems is unknown but their mathematical properties have been partly elucidated. Some of them are found to involve the transmission of morphogenetic impulses while others may be purely local in their action. As one of the consequences of this physiological situation it is found that shoots having opposite directions of phyllotaxy differ quantitatively as well as qualitatively.

INTRODUCTION

IN the quantitative investigation of morphological problems a special place will always be taken by those quantities which, being determined by counting rather than by measurement, express themselves only as a range of positive integers. The holly leaf, with its characteristic marginal prickles, is an almost ideal object for an essay in this branch of morphology, and it is amazing that nothing has been done to exploit its obvious potentialities. The present paper is no more than a preliminary exploration, but it is already clear that a most complex system of relationships exists, and that it is possible to recognize by purely mathematical analysis a number of physiological factors affecting the development of the leaf.

Taking an individual holly leaf, the first step will be to make an arbitrary distinction between its two edges, using the convention established in Fig. 1, which represents a plan view of a leaf and its parent stem. The numbers of prickles on the two edges can then be counted separately, ignoring altogether the terminal spine, which, as it is constantly present, need not be particularly recorded. The two counts being denoted by R and L for right and left edges respectively, the total number of prickles on the leaf would be $(R+L+1)$, a number which plays no further part in this discussion. We shall be concerned instead with the behaviour of the following five numbers:

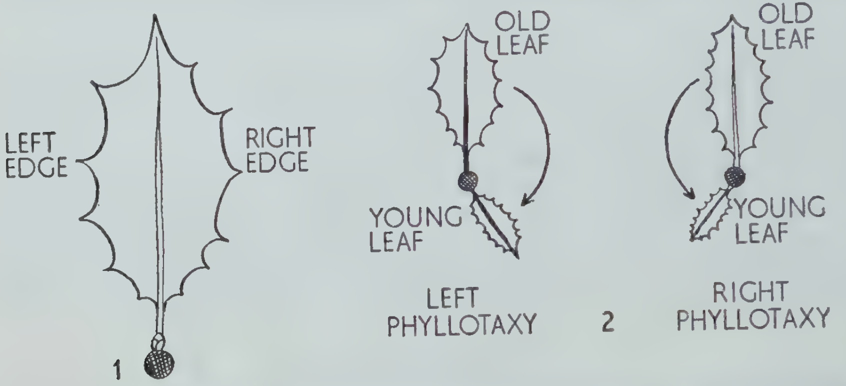
R
L
$(R+L)$
$(R-L)$
$|(R-L)|$

[Annals of Botany, N.S. Vol. XXI, No. 83, 1957.]

the last of these being the difference taken as a positive number, irrespective of sign.

It will be necessary also to take account of the serial number of the leaf, that is its position in the sequence of leaves along the shoot, of the direction of the phyllotactic spiral, and of some measurements of the lengths of leaves and internodes.

The observations were taken in four localities, at Taxal, Mow Cop, and Alderley Edge, all near Manchester, and at Ivybridge in Devon. The unit of material for collection was the growth-increment, that is the length of new shoot expanded during a single growing season. In order to eliminate some of the more obvious causes of heterogeneity the following rules were observed in the gathering of material:



FIGS. 1 and 2. Conventions for distinguishing between the two edges of a leaf and between the two directions of phyllotaxy. All structures are viewed from above.

1. All material was taken between 4 ft. and 5 ft. above ground level.
2. All growth-increments examined were those developed during the summer of 1953.
3. The selected growth-increment was never the first year's growth of a shoot-axis; that is to say all the material was the produce of terminal buds, not lateral ones.

Each growth-increment begins with four or five short internodes associated with scale leaves. This basal portion was entirely neglected, and in computing n, the serial number of a leaf, the first *foliage* leaf was the first to be counted. Great care was taken to avoid errors arising from the accidental loss of leaves. In this connexion it is fortunate that the scar left by a foliage leaf is readily distinguishable from that of a bud-scale. The lengths of leaf and internode were measured in millimetres, leaf-length including the petiole, internode-length being measured between homologous points on successive leaf bases.

The direction of the phyllotactic spiral was determined by applying to the short-way genetic spiral the ordinary convention used by engineers in connexion with right- and left-handed screw threads. An illustration in botanical terms is shown in Fig. 2, which represents a plan view of two shoots, each with two successive leaves.

EFFECTS OF SERIAL NUMBER

The first step in analysing the data is to examine the way in which leaf and internode length and the general spininess of the leaf are related to position on the annual growth-increment. The results of this investigation are presented graphically in Fig. 3, where leaf-length, internode-length and $(R+L)$ are plotted against n, the number adjacent to each point being the number of separate observations contributing to its position. These numbers fall off as n increases, because many shoots have only a small total number of internodes. It is necessary therefore to use some caution in interpreting these graphs, since the higher serial numbers are represented only by shoots which have, in a sense, been selected for their greater vigour. For this reason it has been thought undesirable to extend the graphs beyond the tenth leaf, because to do so would involve the use of points based on less than fifty observations.

It seems to be clearly established that the lengths of internode and leaf show an increase followed by a decrease as the value of n increases. This result is wholly consonant with many other recorded observations of the same kind, and is therefore of no special interest in itself. Two other facts appear, however, which could not have been foreseen. Firstly the cyclic changes of internode length and leaf length are out of phase by about one internode. Secondly the changes of leaf and internode length are *not* associated with any corresponding variation in $(R+L)$; indeed there is no satisfactory evidence that spininess depends on position at all. Although no formal statistical analysis has been attempted these conclusions can be regarded as reasonably well authenticated, for sections of the data, whether arbitrarily chosen or segregated according to locality, display the relationships quite consistently.

RELATIONSHIPS BETWEEN SPININESS AND LEAF SIZE

The discovery that spine number is so largely independent of leaf size is a surprising one, and merits further examination. Quite apart from the size-variation which is due to the sequence of leaves within the annual growth-increment, and which has been shown to have no pronounced effect on spininess, there will also be variation between different specimens; under the conditions of the present investigation it is not possible to say how the responsibility for this heterogeneity of the material is to be apportioned between hereditary and environmental factors.

In order to detect any correlation between spininess and this second, non-sequential class of size-variation, we proceed as follows. It is evident from Fig. 3 that over the range $n = 2, 3, 4$ the mean leaf-length and the mean value of $(R+L)$ are both substantially constant. To be precise, the leaf-length varies by about 2·3 per cent., $(R+L)$ by only about 0·74 per cent. Let us pool all the data within this range and calculate the mean length for all leaves with each possible value of $(R+L)$. The resulting graph is shown in Fig. 4, which clearly reveals a positive correlation between spininess and length. The data are reasonably well fitted by the broken line, which represents the equation:

$$\text{Length} = 46{\cdot}5+2(R+L)$$

In this respect there is some difference between localities. Fig. 5 shows the data for Taxal and Mow Cop plotted separately, the Taxal leaf being slightly longer for a given number of spines.

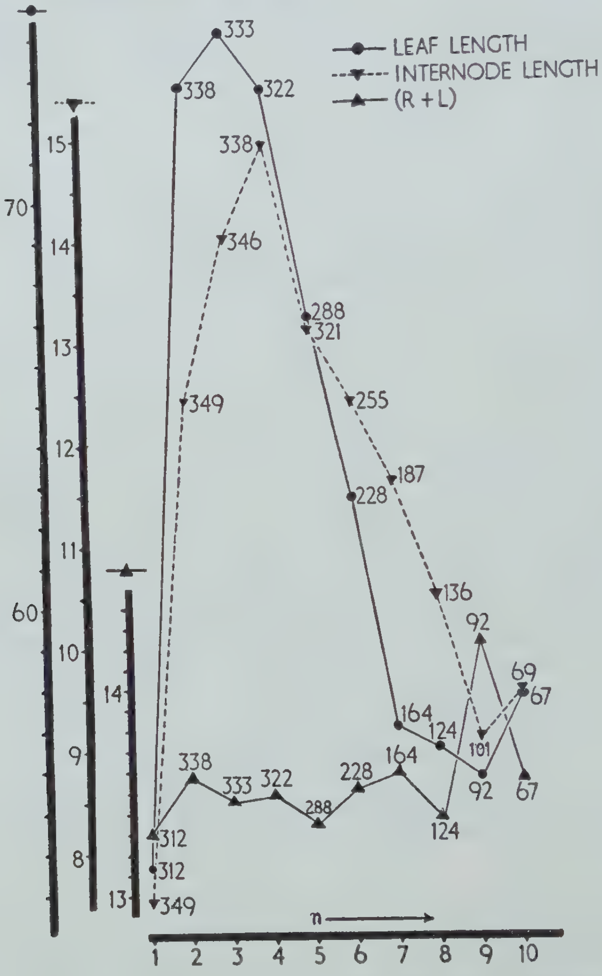
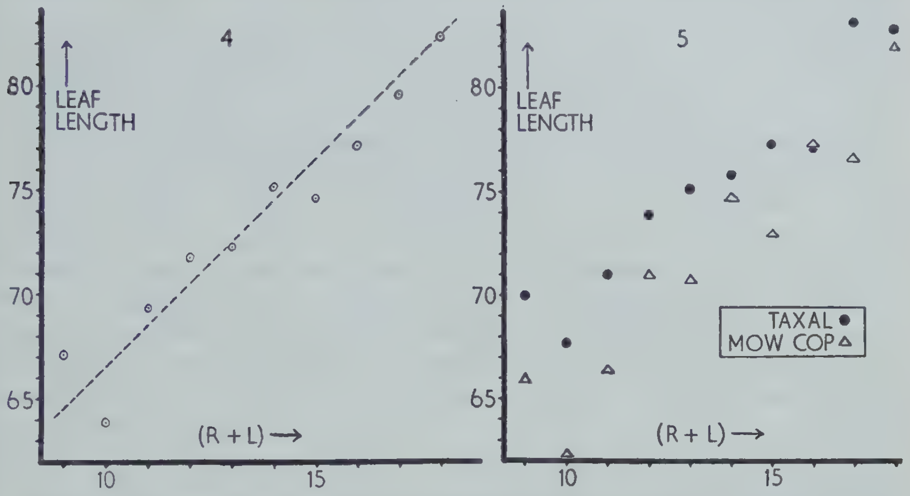


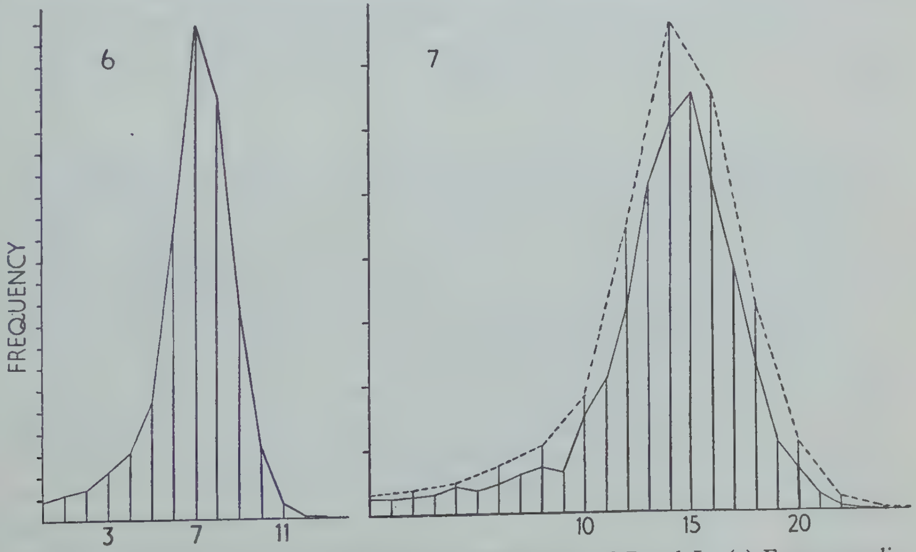
FIG. 3. Graphs of leaf length, internode length, and $(R+L)$, all plotted against n.

FREQUENCY DISTRIBUTIONS

Fig. 6 shows the relative frequencies of occurrence of different values of R or L in the total available catalogue of 8,438 individual counts. By pooling the data in this way we are ignoring any influences which may arise from the distinction between the two edges of the leaf or from the direction of the phyllotactic spiral. Although such influences undoubtedly exist they produce only trifling variations in the form of the graphs which are now to be considered, variations which may quite properly be neglected in the first instance.



Figs. 4 and 5. Relationships between leaf length and $(R+L)$. Data cover the range $n = 2, 3, 4$. The broken line in Fig. 4 represents the equation: Length $= 46{\cdot}5+2\ (R+L)$



Figs. 6 and 7. (6) Frequency distribution of observations of R and L. (7) Frequency distribution of $(R+L)$. Broken line represents data of Fig. 6 replotted on alternate ordinates

The distribution shown in Fig. 6 is unimodal and strongly unsymmetrical, a form of curve which is not in any way unexpected in the circumstances.

The continuous line in Fig. 7 is a frequency curve for the observations of

$(R+L)$. Here again the effect of phyllotaxy is so trivial that the data can legitimately be pooled, and again the distribution is unimodal and unsymmetrical.

Obviously the graph of $(R+L)$ is not entirely independent of the distribution shown in Fig. 6. Suppose that there were a general tendency for R and L to be approximately equal. Then the form of the graph for $(R+L)$ would be approximately that obtained by replotting the data of Fig. 6 on alternate ordinates only, with a suitable reduction in scale. The broken line of Fig. 7 is obtained in this way, reducing the vertical scale to one-third. There is sufficient resemblance between this curve and the observed distribution of $(R+L)$ to establish that there is an approximate equality between R and L. If, however, there were any appreciable tendency for R and L to be *exactly* equal, the distribution of $(R+L)$ would show a predominance of even numbers. In fact nothing of the kind can be detected, even by the most searching statistical tests, and indeed the modal value of $(R+L)$ is itself an odd number.

This work therefore suggests that the two edges of a leaf behave to some extent independently; no mechanism is at work to equalize R and L, nor is there anything to make them conspicuously or regularly unequal.

RELATIONSHIPS BETWEEN SUMS AND DIFFERENCES

The difference between two positive integers cannot exceed their sum, and the extent to which a leaf can display a difference between its two edges will therefore be limited by the total number of prickles available. This consideration leads to the expectation that one of two alternative conditions might prevail:

(*a*) If the inequality of R and L were subject to some overriding limitation, additional to the purely arithmetical one, the mean value of $|(R-L)|$ would be constant for all values of $(R+L)$ exceeding a certain critical value.

(*b*) If the inequality of R and L were not limited by any other factor than the total availability of prickles, the value of $|(R-L)|$ would increase continually with increase of $(R+L)$.

The investigation of this problem is a simple matter, but it will be necessary to deal separately with odd and even values of $(R+L)$. $|(R-L)|$ is a discontinuous variable which will be even or odd according as $(R+L)$ is even or odd. Botanically speaking there is no way of directly comparing odd and even differences. Taking, for example, a leaf with $|(R-L)| = 3$, it is clear that a leaf with a similar degree of inherent asymmetry but with $(R+L)$ an even number would be most likely to have $|(R-L)| =$ either 2 or 4, but there are no grounds upon which to base an estimate of the relative probabilities of these two alternatives.

Once more it is unnecessary to separate the data according to phyllotaxy, so that we have only to calculate for all the leaves with each value of $(R+L)$

the mean value of $/(R-L)/$ and plot two graphs, one for even and one for odd numbers.

The results are shown in Fig. 8. That the mean differences are regularly smaller for even sums is only natural, for an even sum admits of the possibility of a zero difference, whereas an odd sum cannot be associated with a difference of less than one. It is interesting, however, that the separation between the two graphs should be so small, averaging only about 0·318 of a prickle. A thorough investigation of this point would require a more detailed analysis than the available data will permit, but the general implication seems fairly clear. What may be called the 'accidental' difference between the two edges (i.e. the unit difference which necessarily arises when $(R+L)$ is odd) is usually *not* added to any difference arising from other causes, but is opposed to it. In other words a leaf with $R = 12$, $L = 14$ is in a condition which, on the addition of one further prickle, is much more likely to become $R = 13$, $L = 14$ than $R = 12$, $L = 15$. This form of statement implies that the asymmetry of a leaf is determined before the total number of prickles. It may be that the opposite is true, in which case we may adopt an alternative form of statement, saying that an asymmetrical tendency sufficient to bring about a $(12+14)$ distribution of prickles would probably be insufficient to produce the greater degree of asymmetry required for $(12+15)$, and would therefore have to be satisfied with $(13+14)$.

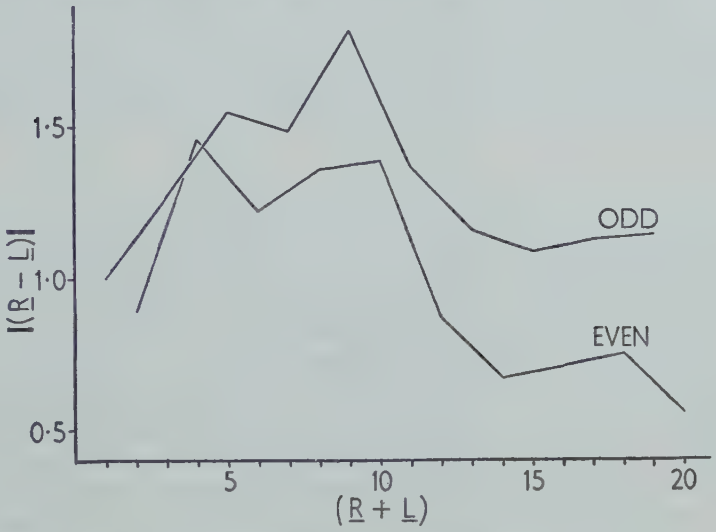


FIG. 8. Mean values of $/(R-L)/$ plotted against $(R+L)$.

A more important feature of Fig. 8 is the occurrence of maximum differences in the range $(R+L) = 4$ to 11, and of much smaller mean differences for higher values of $(R+L)$. It is inevitable that the differences should at first increase with increasing values of $(R+L)$, for with small numbers of spines large differences are arithmetically impossible, but the subsequent decrease of the differences with further increase in $(R+L)$ could not have been foreseen. It seems to be impossible to construct any rational explanation of this phenomenon which does not embody in some form the concept of potential, as distinct from actual, values of R and L. Suppose that some inherent characteristic of a leaf imposes definite limits on the possible magnitudes of R and L. Then when the spinier edge of the two has reached this limit the addition of a further spine can only take place on the other edge, so reducing the difference. An hypothesis of this kind also makes it possible to attach a meaning to the asymmetry of the frequency distributions shown in Figs. 6 and 7. Here the excess of small counts may be attributed to the existence of a fraction of leaves which, for one reason or another, have failed to produce the number of spines which they were potentially capable of generating.

SPINE DISTRIBUTION IN RELATION TO PHYLLOTAXY AND FOLIAR ASYMMETRY

In order to investigate the effect of phyllotaxy on the arrangement of spines it is desirable to examine the distribution of the difference $(R-L)$. The observed values of $(R-L)$ range in a roughly symmetrical manner from $+7$ to -7, with a single conspicuous mode at $(R-L) = 0$. The most sensitive conceivable test for phyllotactic effects will be to compare the whole distribution of $(R-L)$ for shoots of right phyllotaxy with that for shoots of left phyllotaxy. As the exact form of the individual distributions is not of special interest, we shall merely plot the discrepancies between them, as calculated in Table I, upper half. The resulting graph appears in Fig. 9 (continuous line), and is very unsymmetrical, indicating quite unmistakably that inequalities in spine number bear a definite relationship to the direction of phyllotaxy.

The question now arises whether the two forms of phyllotaxy are, as is generally supposed, merely opposite in direction, or whether they may not be different also in other respects. To settle this point we must *reverse* the whole distribution of $(R-L)$ for one direction of phyllotaxy, repeat the subtractions, and plot another graph. The working is shown in the lower part of Table I and the results appear in Fig. 9 (broken line), from which it appears that the asymmetry is now more pronounced than before. This result, which had not been foreseen, is of considerable theoretical importance, for it seems to be the first observation to indicate that the two forms of phyllotaxy are not merely geometrical opposites but things with a difference in quality which is quite independent of stereoisometric considerations.

Although the study of $(R-L)$ is the most sensitive method for detecting the existence of a phyllotactic effect it is not well adapted to the analysis of the different factors involved. For this purpose it will be necessary to examine in more detail the distributions of R and L considered separately. The frequency curve for the pooled data has already been given in Fig. 6, and the observations contributing to this curve can be divided into four classes: R (right phyllotaxy), R (left phyllotaxy), L (right phyllotaxy), and L (left

TABLE I

(*R–L*)	−7	−6	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5	+6	+7
Right phyllotaxy	1	1	5	23	53	182	445	665	487	148	34	17	7	—	—
Left phyllotaxy	—	3	5	21	36	137	426	726	575	186	43	17	5	4	1
Differences	+1	−2	0	+2	+17	+45	+19	−61	−88	−38	−9	0	+2	−4	−1
Right phyllotaxy	1	1	5	23	53	182	445	665	487	148	34	17	7	—	—
Left phyllotaxy reversed	1	4	5	17	43	186	575	726	426	137	36	21	5	3	—
Differences	0	−3	0	+6	+10	−4	−130	—61	+61	+11	−2	−4	+2	−3	—

phyllotaxy). The frequency curve for any one of these classes will differ very slightly in form from the graph of Fig. 6, and these differences can conveniently be magnified by plotting the four sets of averages as positive or negative deviations from the curve of Fig. 6. The resulting graphs are shown in Fig. 10.

Although these curves are very irregular in detail, it is evident that the basic form is the same for all four, and that all the data can be fitted reasonably well by the use of a single template, which the reader may trace, if he chooses, from the shape given in Fig. 11. This shape was derived from the data of Fig. 10 by a crude averaging procedure, and may be adapted to any of the four graphs of Fig. 10 by turning it over and moving it from side to side.

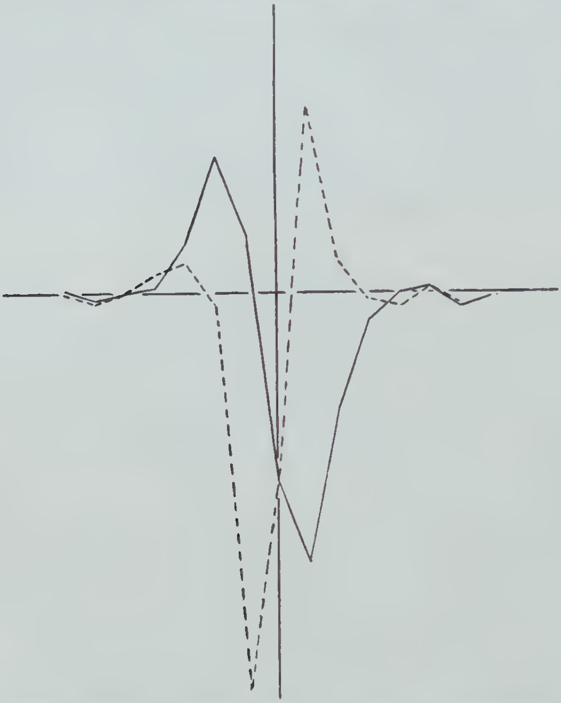
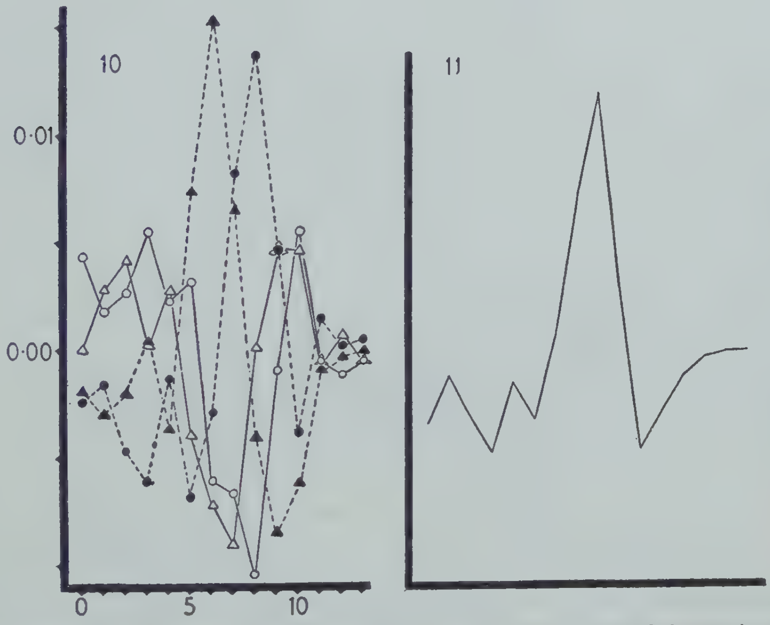


FIG. 9. Unsymmetrical graphs demonstrating the effects of phyllotaxy. For explanation see text and Table I

In Fig. 10 the difference between right and left phyllotaxy is represented by an inversion of the graphs, which signifies that right phyllotaxy is associated with a greater variability in the spine counts, left phyllotaxy with a higher degree of standardization. This result confirms the impression that the two forms of phyllotaxy are not merely mirror-image opposites.

The difference between the two edges of a leaf manifests itself in Fig. 10 as a lateral displacement of the graphs. So far as the scope of this investigation is concerned, the right edge of a leaf behaves as though it had slightly fewer spines than is actually the case, the left edge as though it had slightly more.

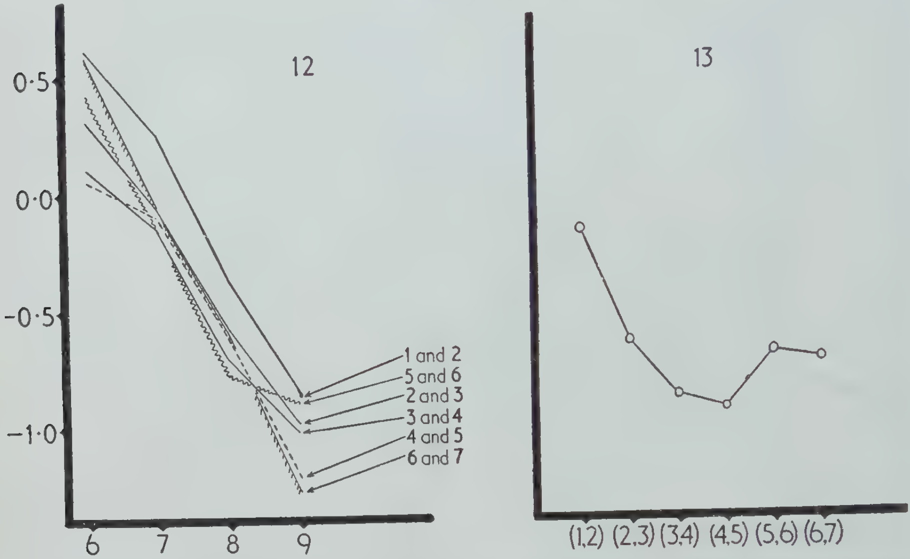
Botanically speaking, this result could arise in either of two ways, between which it is at present impossible to distinguish. The first alternative is that the development of spines is a response to some kind of applied potential, and that the sensitivity is different for the two edges. The second possibility is that the spines, by their presence, produce some reaction upon the leaf, and that the strength of this reaction for a given number of spines is different in the two edges.



FIGS. 10 and 11. (10) Frequency distributions for *R* and *L*, the four sets of observations taken separately and plotted as departures from the mean. Circles refer to right margins, triangles to left; white symbols and continuous lines refer to right phyllotaxy, black symbols and broken lines to left. Scale of ordinates in absolute proportions. (11) Template graph, the shape of which can be roughly fitted to any of the four graphs of Fig. 10.

RELATIONSHIPS BETWEEN ONE LEAF AND THE NEXT

Between the different leaves upon a shoot there must exist very numerous relationships in respect of spine number. It would theoretically be possible to examine the correlation between any one spine-count and any other on the same piece of material. In fact the data of the present paper are insufficient to support any extended investigation of this kind. All that it has been thought worth while to attempt is an assessment of the correlation between one spine-count and that taken on the corresponding edge of the next leaf. The data have not been segregated according to phyllotaxy or leaf symmetry. The procedure has been to calculate from the pooled data the average number of spines which follows any given number. It is found, for example, that if the first leaf of a growth-increment has 6 spines on one edge, the number of spines on the corresponding edge of the second leaf will be, on the average, 6·622. The values obtained in this way have been used in the construction of the graphs of Fig. 12, where the numbers are shown as excess of spines on the second leaf as compared with the first (i.e. 0·622 in the example just given). Each line represents one correlation, the highest leaf taken into account being number 7, and the spine-counts being utilized over the range (for the lower leaf) 6–9. It is only within these limits that there are sufficient data to yield a trustworthy result. There is obviously a displacement of successive curves, first downwards, then up again. By summing the effects over the whole range we obtain the graph shown in Fig. 13. Displacements of this kind have become familiar in quantitative morphological researches on other material (Dormer, 1950, 1951; Dormer and Plack, 1951) and they indicate quite clearly that the shoot does undergo a cyclic system of changes in respect of spine production, although these changes are not of a kind to be revealed by direct examination of the spine counts as in Fig. 3.



Figs. 12 and 13. (12) Relationships between spine number of one leaf and that of the next. Range covers leaves 1 to 7, lower leaf having spine-counts (*R* or *L*) 6 to 9. (13) Curve obtained by summation from Fig. 12, showing how the relationship varies with *n*.

Interpretation

The development of the spines in this species is undoubtedly a very complex process, but from the mathematical study of spine-counts it is possible to arrive at an analysis which separates the effects of some of the principal factors involved. The only thing that can never be deduced from an examination of the mature spines is a statement of the order in which these factors operate.

Taking the results which have been obtained, and setting up the simplest conceivable system of relationships which could account for them, we obtain a synopsis of foliar development in the following form:

(*a*) There exists a mechanism which we may call **P**, which generates in each leaf-margin some kind of physiological potential which is concerned with spine-production, and which is imperfectly standardized as between different leaves. **P**, so far as we know, is entirely internal to the individual leaf, and as it is one of the characteristics of the species it must be genetically determined, at least in part.

(*b*) The action of **P** is modified by another mechanism which may be called **Q** and which is concerned with phyllotactic influences. Some part of **Q** must involve the transmission of impulses into the leaf from external sources, and may possibly be a hormone action. **Q** acts upon **P** by varying the degree of standardization of the potential produced, and there is nothing to indicate that **Q** is subject to any direct genetical control.

(*c*) A third mechanism, **R**, is concerned with asymmetry of the leaf, and there is nothing to show that any part of it is external to the leaf. Although there is no direct evidence, many analogies suggest that **R** will be directly inherited as a stereoisometric property of protoplasm. It is not clear that **R** has any direct effect on **P**, but it certainly modifies the operations of **Q**, either by changing the susceptibility of **Q** to reactions arising from the physiological potential generated by **P** or by varying the sensitivity of the leaf tissues to the resultant effect of **P** and **Q** combined.

(*d*) The potential arising from the joint action of **P**, **Q**, and **R** is one of the factors required for spine-production, but other factors, denoted by **S**, are also needed. The spine-output made possible by the **P**–**Q**–**R** potential will be realized only so far as the availability of **S** permits. Leaves which are conspicuously lacking in spines are mostly those in which there has been, for unknown reasons, a deficiency of **S**. Leaves which have appreciably more than the average number of spines are mostly those in which the **P**–**Q**–**R** system has been the limiting factor.

(*e*) There is no evidence that the output of spines is directly related to n, but the spininess of a leaf is correlated with that of lower leaves in a manner which does vary from one internode to another. Some kind of transmitted impulse must be involved, but it is not possible at present to say how this is connected with the systems **P**, **Q**, **R**, and **S**.

DISCUSSION

The result of this investigation is to show that the number of prickles on a leaf margin is determined by complex reactions between physiological systems which are not at present known to have any other effects. It may be felt that as the product of these reactions is a rather trivial morphological character the physiological mechanisms involved are not likely to be of any great importance. We are not prepared to admit the validity of this argument.

It appears to us to be very improbable that the interacting systems were evolved solely with reference to their effects on the number of prickles, as we are quite unable to attribute any adaptive value to the precise regulation of this character. We therefore regard the number of prickles as merely a convenient indicator revealing the operation of hidden mechanisms which are probably concerned primarily with other and more important matters, and which are likely to be active also in other leaves where there are no spines to inform us of their effects.

It will probably be objected also that a purely mathematical analysis of the relationships between physiological systems contributes nothing to the elucidation of their material basis. Considerations of this kind have not been allowed to hold up researches in genetics or in the physical sciences, and we do not propose to regard them. We are concerned here with the study of the ways in which basic physiological mechanisms are assembled and interconnected to make a plant, and we reject any suggestion that research in this branch of physiology must necessarily wait upon the detailed analysis of the individual mechanisms themselves.

SUMMARY

The number of prickles on the edge of a leaf is found to be governed by a complex series of interactions involving not less than five distinct physiological systems. The physical nature of these systems is unknown, but the relationships between them have been partially elucidated. The result is a scheme of foliar development which is set out in the lettered paragraphs of the section headed INTERPRETATION above.

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Studies in the Growth of Young Plants of *Pinus sylvestris* L.

I. The Annual Cycle of Assimilation and Growth

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With four Figures in the Text

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ABSTRACT

The annual cycle of growth in plants of *P. sylvestris* L. varying in age from 2 to 5 years was studied mainly by following their weight changes through periodic sampling. Immediately following bud-break there was a period of about 1 month in which, although meristematic and extension growth were rapid in both shoot and root, there was no gain in weight by the plant. There was evidence that the needles remaining from previous years contained a considerable amount of storage material which was utilized at this time. During May and June the main increase in weight was in the new shoots, but in August and September assimilates were more evenly divided between the new shoots on the one hand, and the old stem and the root on the other. The average relative growth-rates for the months May to September were between 1·0 and 0·4 per cent. per day in plants growing in favourable conditions, the lower rates being found in older plants. During the months October to March slow increase in weight occurred in needles, stem, and root, and the R.G.R.s in two experiments were 0·21 and 0·15 per cent. per day. The net assimilation rate fell with increasing age and in the summer of the fifth year was 0·06 g./g./week or 0·10 g./dm.^{2}/week. This low value of N.A.R. is discussed in relation to the known high rate of production of *P. sylvestris* stands and their evergreen habit.

INTRODUCTION

THE growth of many annual and herbaceous perennial plants has been exhaustively studied by the techniques of growth analysis conceived by Gregory (1917) and Briggs, Kidd, and West (1920), techniques which depend

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on taking periodic samples from a given population of plants in order to determine their rate of change in dry weight, and the way in which the change is distributed between the various parts of the plant. Studies of the growth of trees, on the other hand, have for obvious practical reasons been mainly restricted to the measurement of increase in height, girth, and volume of timber; and periodic sampling for the determination of weight increment has rarely been carried out after the end of the first or seedling year. There are, however, a few examples of the application of growth-analysis techniques to older trees.

In order to follow the uptake of nutrients by a number of coniferous species, Bauer (1910) took samples throughout a growing season of plants varying from 2 to 4 years old according to the species. In the case of *P. sylvestris* he took five samples of 250 plants in their third year of growth, divided them into old and new needles, stem, and root, and recorded the dry weights of the parts as well as their chemical composition. It is uncertain how far his samples fulfilled the condition of randomness, or what was the sampling error, but his data have been used later in this paper to calculate net assimilation rates. Burger, by fellings in forest stands in Switzerland, calculated the relation between the annual increment of timber and the weight of foliage for many species, including *P. sylvestris* (1941, 1948), and investigated the variation of this relation not only between one species and another, but also with differences in seed origin, environment, especially altitude, and age. Ovington (1957) has constructed a picture of growth and weight change of all parts of the tree for the first 50 years of its life by felling representative trees and digging out the roots in stands of *P. sylvestris* varying widely in age, but growing in the same soil and climatic conditions. In the horticultural field, Vyvyan (1955) has published the first paper in a series in which the interrelation of scion and rootstock in apple-trees has been examined by growth-analysis methods, and this same problem has been approached in a different way by Ruck and Bolas (1956), who have compared the N.A.R. of different rootstock varieties.

At the outset of investigations into the ecology and physiology of *P. sylvestris* it was felt that an analysis of the annual cycle of growth and weight change was of basic importance. A considerable literature exists on development and growth in size of *P. sylvestris* and other species of pine, and for this reason less attention has been given in the present investigation to these aspects of growth, and more to the measurement of weight change and the calculation of relative growth-rate and N.A.R. It is true that a number of thorough studies of assimilation and respiration at different times of the year and under different environmental conditions have already been made by gas-exchange methods for the species under consideration. But the value for ecological purposes of an estimate of net assimilation over periods measured in days or weeks, and the difficulties and errors attendant upon making such an estimate from gas-exahange experiments lasting for periods of an hour or less are too well known to need elaboration. Nevertheless, growth-analysis techniques constitute only one of the many approaches to the study of growth and development, and the literature now only indirectly alluded to will be more fully cited as the present paper is developed, and as an attempt is made to build up a more comprehensive account of the annual cycle of growth in *P. sylvestris*.

The task of taking periodic samples of adequate size from a population of fully grown trees within one growing season would demand exceptional facilities and the present work has of necessity been restricted to plants up to 5 years of age. The danger of drawing conclusions from these experiments about the growth of older trees is fully recognized but it seemed reasonable to believe that by the fourth year, when the trees carry 3 years' growth of needles, their organization is qualitatively, if not entirely quantitatively, similar to what it will remain at least until the closure of the canopy.

P. sylvestris is a species which grows, and has been experimented with, under a wide range of climatic conditions, and consequently the average conditions (between 1949 and 1953, the period during which the present experiments were carried out) are indicated in Table I.

METHODS

1. *Sampling*. In most of the experiments to be described populations of plants of uniform age, varying from 2 to 5 years, were sampled at approximately monthly intervals during April to September, and in one experiment at 2-monthly intervals during October to March. The size of the samples varied from 20 to 50 plants, but was constant within any one experiment. The roots were extracted from the soil as completely as was possible without recourse to sieving large volumes of soil, but many of the finer roots must have been lost.

The total sample on any one occasion consisted, depending on the experiment, of from 4 to 8 replicate groups of plants. In the early experiments each replicate was taken from a different area or 'block' of the ground occupied by the whole population. But it was soon realized that the size differences between individual plants did not correspond at all closely to 'block' differences. In later experiments, therefore, a sufficient number of plants to provide all the samples required from the population was chosen at random and marked or transplanted at the beginning of the experiment. These plants were then divided into a number of size classes corresponding to the desired number of replicates. The division into size classes was usually made by measurements of hypocotyl diameter, since it had been found (Rutter, 1955) that this measurement is closely related to the dry weight of plants of *P. sylvestris*. The replicate samples on any one occasion were then each obtained from a distinct size class. In this way the errors of comparison between one occasion and the next were much reduced. The alternative method of reducing the errors, namely by taking larger samples, was impractical both because of the work involved in dealing with them and the limitation of oven space.

TABLE I

Average Monthly Climatic Factors for the Region of the Experiments

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Mean temp. °C	4·0	4·1	5·7	7·9	11·3	15·0	16·9	16·3	13·5	9·9	6·2	5·0
Precipitation, mm.	26	64	43	53	50	53	51	71	59	62	94	38
Evaporation, mm.	< precipitation			76	99	124	126	98	61	< precipitation		
Noon light intensity, lumens/sq. ft.	805	1,200	1,775	2,275	2,790	3,015	3,180	2,990	2,210	1,700	940	700

Temperature and precipitation: Silwood Park, Berks., 1949–53.
Evaporation (estimated from other meteorological measurements): South Farnborough, Hants, 1949–53, supplied by Meteorological Office.
Light intensity: Teddington, Middx., 1933–9 (McDermott and Gordon-Smith, 1951).

After sampling, the plants were washed, root and shoot separated, and the shoot divided into stem, and needles where present, of each year's extension growth. The material from each replicate, but not from each individual plant, was kept distinct and was dried to constant weight at about 80° C. in an oven with moving air-stream, and weighed.

2. *Needle area and volume.* In several experiments the relationships between needle weight, volume, and area were measured. The method used was similar to that used by Blackman and Rutter (1948) for the leaves of *Endymion nonscriptus.* Treating each needle age separately, segments of standard length were cut from a random sample of needles and their breadths measured under a microscope with an eyepiece micrometer. The measured samples were then dried and weighed, and factors relating weight to area or volume were calculated from each age of needle.

Volumes were calculated from length and breadth on the assumption that the segments were half-cylinders. In order to derive N.A.R. per unit leaf area, needle areas could be expressed in two different ways, namely either as the projection on to a plane surface

$$l \times b,$$

or as the total surface of a half-cylinder

$$l \times b\,(1+\pi/2),$$

where l and b are the length and breadth respectively of a measured needle segment. The first expression represents the maximum effective area of light interception, and the second the area available for the entry of carbon dioxide into the leaf, since stomata are found on all surfaces. These two areas are in the ratio of approximately 1:2·57, and N.A.R. calculated from them would be in the inverse ratio. N.A.R. in this paper has been calculated on the basis of the first method of expressing area, solely in order to make data more comparable with those from plants with flattened leaves, in which the term 'leaf area' has usually meant the area of one side only of the leaf.

3. *Starch content of needles.* In a few experiments needles were sectioned and examined for starch with iodine. The starch contents were divided into the following arbitrary grades:

0·5	Grains sparse, in stele only.
1·0	Grains dense in stele; sparse in one-fifth of mesophyll.
1·5	Grains sparse in stele, and throughout the mesophyll.
2·0	Grains dense in stele; sparse throughout mesophyll.
2·5	Grains sparse in stele; dense in one-quarter of mesophyll.
5·0	Grains dense in stele and in half the mesophyll.
7·5	Grains dense in stele and in three-quarters of the mesophyll.
10·0	Grains dense in stele and throughout the mesophyll.

In the stele starch grains were found in the endodermis and the ground parenchyma. By sparse is meant that they were separated from one another in the cells, and by dense that they mostly touched one another. Although the lower grades appear somewhat unsystematic, they correspond to the types of starch distribution most commonly noted. The scheme is obviously capable of some subdivision.

In one experiment the stem also was examined for starch and a similar scale constructed. However, since it was not widely used or tested, the details of the scale will not be given here; starch was found in the pith, the phloem rays, the secretory cells of resin canals, and the cortex.

RESULTS

1. *Development and linear measures of growth*

Extension of the buds is first seen at about the beginning of April and continues for 3 to 6 weeks before extension of the needles beyond the tips of their subtending scales is noted. Shoot extension is virtually complete by the second half of June, but needle extension continues until at least the end of July or early August. The basal (hypocotyl) diameter has been observed to decrease by a few per cent. in mid-winter and to increase again in February, but rapid radial growth begins at about the time that bud extension is first noted and continues throughout the summer, persisting for 6 to 8 weeks after appreciable needle growth has ended, i.e. until early October.

The progress during the season of these three linear measures of growth is shown in Fig. 1, where the lengths of the leading shoot and needles are plotted as percentages of their final lengths, and the diameter increments as percentages of the total increment in 12 months. These observations were obtained from successive samples of 48 plants in their fourth year of growth in Experiment II (which is to be described), but since the changes of basal diameter were not followed during the winter in this experiment, the figure has been supplemented by data obtained in 1952–3 from a set of 40 plants in their fifth year of growth, whose diameters were measured periodically at a marked point at the base of the stem.

Other series of observations, similar to those shown in Fig. 1, were obtained, but since the main object of these experiments was to follow weight change, and since the measurement of successive samples is an unnecessarily crude method of following linear growth, it has seemed sufficient to present the foregoing selected data which closely resemble observations made on leader growth of *P. laricio* by Hiley and Cunliffe (1922), and on leader, needle, and radial growth of *P. resinosa* by Kienholz (1934).

Kienholz found no increase in length of the second-year needles of *P. resinosa* or *P. strobus*, but he refers to earlier papers which claimed that the second-year needles of *P. radiata* and *P. palustris* may increase in length. Freeland (1952) also states that he found the volume of the needles of a number of conifers including *P. sylvestris* to increase from year to year, but he gives no indication of the magnitude of the increase, nor whether it occurred in length, breadth, or both. His conclusions were not reached from periodic measurements on the same needles, but by simultaneous measurements on needles of different age. In Experiment II, from which the data for Fig. 1 were drawn, the length and breadth of the second-year needles on the main axis were measured at each sampling. Neither measurement showed any variation with time greater than the sampling error which was about 2·3 per cent. of the length and 0·7 per cent. of the breadth.

The cycle of development within the bud has been described in detail by Sacher (1954) for *P. lambertiana* and *P. ponderosa*, and I am grateful to Mr. E. R. C. Reynolds for allowing me to use observations which he made on *P. sylvestris* in 1955 for comparison with Sacher's study. The differentiation of scale leaves by the apical meristem begins at or shortly after bud-break and continues until some time in September. Neither the earliest- nor the latest-formed scales ever develop axillary structures, but axillary buds—the dwarf shoots of the following year—are differentiated from the end of June to the middle of August. Initially there is a lag in this axillary development, and the first dwarf shoot primordia appear in the axils of scales which were formed some time previously. By August, however, axillary buds appear almost immediately after their subtending scales. During the last month of activity, when only a few scales are produced at the apex, the later-formed axillary buds increase in size until they are equal to the earlier. The uppermost axillary buds, which will ultimately form branches, are at this stage difficult to distinguish from the short shoot primordia.

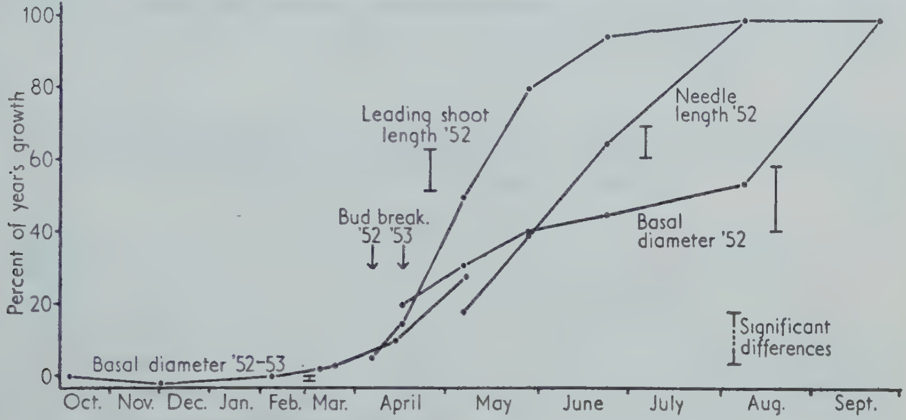

FIG. 1. The seasonal course of height, radial, and needle growth of *P. sylvestris*.

Wareing (1951) observed that all bud growth of *P. sylvestris* appeared to have ceased by early September in southern England, but, at least in 1955, Reynolds noted a marked change in the size of the upper axillary buds between August 29 and September 22.

2. *Growth in weight and expansion of needle area from April to September on fertile loam soils* (Experiments I and II)

After a description of the conditions of these experiments, their results can be considered together. It should be noted that the experiments are not numbered in chronological order.

Experiment I was carried out in 1953 on plants in their third year which had been transplanted at the end of their first and second years. For their age these plants were rather small and poorly branched. They were grown during the experiment in a sandy loam, kept free from weeds, at Silwood Park, Berks. The plants were separated from one another by about 30 cm., a spacing at which inter-plant competition was probably negligible. Each sample consisted of 8 replicate sets of 4 plants. When the first sample was taken on April 20, very few buds showed signs of growth. At the second sampling, May 26, the apical shoots had grown to about 5 cm. length—their final length was about 11 cm.—but very few of the needles had extended sufficiently for their tips to project beyond the subtending scales.

TABLE II

Experiment I, 1953. Seasonal Changes in Dry Weight and Relative Growth-rate of Plants in their Third Year of Growth

	Means of 32 plants					Sig. diff. ($P =$ 0·05)
	Apr. 20	May 26	June 25	July 30	Sept. 11	
Dry weight per plant, g.						
1953 needles .	—	—	0·80	3·88	6·19	—
1953 stem . .	—	—	0·38	0·79	1·56	—
Total new growth .	0·07	0·29	1·18	4·67	7·75	—
Old needles . .	1·48	1·15	1·10	1·24	1·04	0·21
Old stem . .	0·94	1·07	1·07	1·64	2·62	—
Root . . .	0·79	0·80	0·86	1·54	2·65	—
Whole plant. .	3·28	3·31	4·21	9·09	14·06	—
Log. dry weight of 100 plants						
New growth. .	0·773	1·407	1·982	2·638	2·828	0·150
Old stem . .	1·946	1·988	1·999	2·154	2·374	0·059
Root . . .	1·877	1·857	1·896	2·164	2·386	0·083
Whole plant. .	2·497	2·482	2·584	2·931	3·098	0·080
Relative growth-rate, per cent. per day		−0·20	1·18	2·15	0·89	1·15

Experiment II was carried out mainly in 1952, on plants in their fourth year. These plants were germinated and grown in a silty loam at Slough, Bucks., infertile by agricultural standards but on which the growth of *P. sylvestris* was vigorous. They were transplanted at the end of their first year and thinned at the end of their second. When the experiment began at the end of their third year they were spaced 30×45 cm. apart and had not been disturbed for the two preceding years. Their height was then 40 cm., and during the following season it increased to 72 cm. At the spacing stated such tall plants were no doubt in competition with one another to some extent, although the progressive decrease in density, brought about by sampling, was such that they were on average always separated from one another by a distance similar to their height. For this reason and because the soil, kept free of weeds, was not at all densely exploited by the pine roots, it is considered that competition played a relatively unimportant part in the development of these plants. The samples consisted of 4 replicate sets of 12 plants. The first sample was taken in October 1951 and the second on April 16, 1952, when the buds had been growing visibly for about 10 days. The third sample, May 6, resembled that of May 26 in Experiment I, in that the leading shoot had grown to approximately half its final length, and that there was practically no visible needle extension. Thus the April and May samples of Experiments I and II were chosen to cover the same stage of development, but this stage took longer in Experiment I. This may have been because the plants of Experiment I were transplanted in the February preceding the beginning of the experiment, or because the mean air temperature in April 1953 was 7·8° C. and remained at about this value until May 10, whereas the mean air temperature in April 1952 was 10° C. rising to about 12° C. in early May.

TABLE III

Experiment II, 1951–2. Seasonal Changes in Dry Weight and Relative Growth-rate of Plants in their Fourth Year of Growth

	Means of 48 plants							Sig. diff.
	1951 Oct. 9	1952 Apr. 16	May 6	May 27	June 23	Aug. 8	Sept. 23	(*P* = 0·05)
Dry weight per plant, g.								
1952 needles	—	—	—	14·4	44·4	79·7	102·3	—
1952 stem	—	—	—	15·9	21·4	25·5	44·1	—
Total new growth	—	3·9	11·1	30·3	65·8	105·2	146·4	—
Old needles	50·3	71·7	59·7	67·6	59·5	47·3	32·8	11·2
Old stem	38·6	53·2	53·7	65·0	61·5	74·7	98·6	—
Root	20·4	35·7	32·7	33·7	30·1	38·1	60·7	—
Whole plant	109·3	164·5	157·2	196·6	216·9	265·3	338·5	—
Log. dry weight per plant								
New growth	—	0·586	1·039	1·474	1·816	2·011	2·164	0·104
Old stem	1·586	1·721	1·729	1·811	1·786	1·866	1·991	0·086
Root	1·311	1·546	1·516	1·526	1·476	1·579	1·781	0·074
Whole plant	2·039	2·213	2·197	2·292	2·336	2·418	2·528	0·075
Relative growth-rate, per cent. per day		0·21	−0·18	1·04	0·38	0·41	0·55	

The weight changes in these two experiments are shown in Tables II and III. Except in the case of the old needles, statistical analysis of the weights has been carried out after logarithmic transformation (Fisher and Mackenzie, 1923) because an increase of error with mean weight was observed.

The tables also include relative growth-rates for each period between two successive samples, calculated by the formula

$$r = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_2} \, 100$$

where w_1 and w_2 are the dry weights of the plant at times t_1 and t_2.

Neglecting for the moment the gain in weight between October 9, 1951 and April 16, 1952 in Experiment II, the two experiments can be summarized together. During the initial period of growth after bud-break there was little change in the total weight of the plant, although this was a period of considerable activity, for the new shoots extended to about half their final length, and increased in weight three- or four-fold. This increase in weight was approximately equalled by a significant decrease in the weight of the old needles.

Subsequent samples showed a progressive increase in plant weight up to the middle of September when the experiments were ended. When this increase in dry weight is analysed it is found that the new stems and needles increased steadily in weight, this increase continuing unchecked long after extension growth of both these parts had ceased. The increase in weight of the older parts of the stem was rather slow and barely significant in April to June, but more rapid in July to September, and this pattern of growth was even more marked in the roots, in which there appeared to be no increase in weight until at least the end of June. In the case of Experiment II some doubt was felt as to whether recovery of the roots from the soil had been sufficiently complete to make this result reliable, since the plants were large and the soil often dry and somewhat caked. But recovery of the roots of the small plants of Experiment I appeared to be satisfactory, and since the two experiments gave essentially the same picture of weight change in the roots, it is probably reliable. The period during which little change in root weight was detected has been observed to be one of rapid root extension. The striking change, as the season progressed, in the partition of dry-weight increment between the new growth and the old is shown in Table IV.

TABLE IV

The Ratio of the Weight Increase of New Growth (Stem and Needles) to the Weight Increase of Old Stem and Root, in Different Periods of the Summer

Experiment I . .	May 26–June 25 15·7	June 25–July 30 2·9	July 30–Sept. 1 1·5
Experiment II . .	May 6–June 23 24·0	June 23–Aug. 8 1·9	Aug. 8–Sept. 23 0·9

After their initial fall in weight, the old needles recovered slightly, but then showed a further decrease. In Experiment II an estimate of the number of old needles was made at each sampling, from the total weight of needles and the weight of 100 taken at random. These estimates have high errors but it may be seen in Table V that in the second part of the season there was a significant fall in the number of second-year needles, and complete shedding of third-year needles. The fall in total weight of old needles is partly explained by shedding therefore, but was also due to a decline in the weight of individual needles, as will be shown.

Not only was there preferential translocation of assimilates to the new stem and needles in the first part of the summer, but the area per unit weight of the needles, shown in Table VI, was initially high. The development of needle area in relation to the growth in weight of the plant is illustrated in Fig. 2.

Until the end of July the ratio of the area of new needles to the total weight of the plant (new-needle-area ratio) increased, i.e. the area of growing needles increased more rapidly than plant weight up to this time. During August and September the ratio fell slightly. This fall coincided with some shedding of older needles so that the total needle-area ratio was at a maximum in June and July and then showed a significant fall.

TABLE V

Experiment II, 1951–2. Seasonal Course of Needle Fall in Plants in their Fourth Year of Growth

Numbers per plant

	1951 Oct. 9	1952 Apr. 16	May 6	May 27	June 23	Aug. 8	Sept. 23	Sig. diff. (*P* = 0·05)
1951 needles	4,010	4,530	4,390	4,960	3,170	3,220	2,110	1,280
1950 needles	89	57	64	53	39	0	0	43

Before commenting on the seasonal variation of relative growth-rates some remarks must be made about the statistical analysis of these data. I am indebted to Dr. O. V. S. Heath for pointing out to me that when, as in these experiments, one sample has to serve as the end of one growth-period and the beginning of the next, the two successive values of relative growth-rate are not independent; for if a sample weight on one occasion is by chance variation low, then the estimate of growth-rate for the preceding period is depressed, while that for the following period is correspondingly raised. In order that only independent estimates be submitted to analysis of variance, Heath suggests that one of the following alternatives must be taken: either the analysis must be carried out on data from which alternate periods are omitted, or else only half the replicates may be used to calculate growth-rates for one period, and

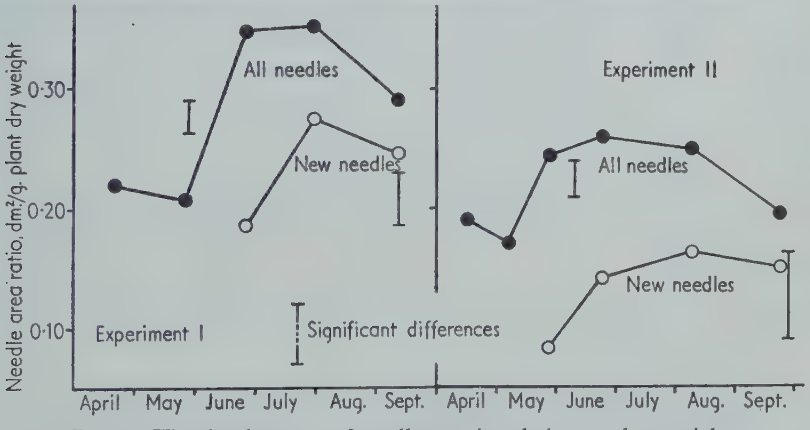

FIG. 2. The development of needle area in relation to plant weight.

the other half for the succeeding one. It should also be pointed out that only growth-rates measured over approximately the same length of time should be included in the same analysis of variance, for the variance of a relative growth-rate is the combined variances of the logarithms of plant weight at the beginning and end of the period, divided by the length of the period; and for longer periods the error is proportionately lower.

In Experiment I, where there were 8 replicates, it has been possible to follow the second of these courses and to show that the relative growth-rate was significantly higher in the middle of the growing season than at either end of it. The mean relative growth-rate from April 20 to September 11 was 1·40 per cent. per day.

TABLE VI

The Relation between the Area and Dry Weight of the Current Year's Needles; dm.^{2}/g.

Experiment I . .	June 25	July 30	Sept. 11	Sig. diff. ($P = 0·05$)	
	1·07	0·65	0·58	0·11	
Experiment II . .	May 27	June 23	Aug. 8	Sept. 23	Sig. diff. ($P = 0·05$)
	1·13	0·70	0·55	0·51	0·11

In Experiment II there were only 4 replicates and neither method of dealing with the relative growth-rates would produce sufficient degrees of freedom for an analysis of variance of the rates during the period of active growth. It appears, however, from Table III that the relative growth-rate was fairly constant throughout the summer. The high value between May 27 and June 23 was due almost entirely to one replicate of high weight on June 23, and may confidently be ascribed to experimental error. The mean relative growth-rate from April 16 to September 23 was 0·45 per cent. per day, about twice as great as the rate during the winter months October 9 to April 16, but less than half the rate of the third-year plants of Experiment I over a similar summer period.

3. *Growth in weight from April to September on a sandy heath soil* (Experiments III and IV)

These two experiments were carried out in natural regeneration of *P. sylvestris* at Bramshill Forest, Hampshire. An area on which a plantation was destroyed by fire in 1945 was ploughed in 1946 in such a way that the ground was thrown up into broad ridges 3 to 4 ft. (90–120 cm.) wide and separated by furrows about 15 in. (38 cm.) wide. The soil was a podsolized stony sand and the ploughing went down to the consolidated B layers which were left exposed in the furrows. In the spring of 1946 there was a dense natural establishment of seedlings on this ploughed ground and marked differences in growth between the plants on ridge and furrow soon appeared. The Ridge plants showed moderate growth for this species; their height after

4 years was 45 cm., and after 9 years 223 cm. The growth of the Furrow plants was very poor, the needles were short and chlorotic, and the branching sparse. Their height growth was the same as the Ridge plants for the first 4 years, but subsequently was much retarded. These two sets of plants seemed representative of moderate and very poor growth respectively of *P. sylvestris* on the infertile sandy soils on which it is often planted. Records of their height and radial growth for 9 years have been presented in another paper (Rutter, 1955).

Two sufficiently large random selections of the Ridge and Furrow plants respectively were marked at the beginning of 1949, and random samples of 20 plants from each marked population were taken during the fourth summer of growth (Experiment III). The increase in weight of the Furrow plants in this experiment was not significant compared with the large sampling errors and is not presented here; four more samples, this time of 40 plants each, were taken from this population between April 1 and September 23, 1950 (Experiment IV).

TABLE VII

Experiment III, 1949. Seasonal Changes in Dry Weight and Relative Growth-rate of Plants in their Fourth Year of Growth

Means of 20 plants

	Apr. 8	May 17	June 27	Aug. 9	Sept. 20	Sig. diff. (P = 0·05)
Dry weight per plant, g.						
1949 needles	—	—	21·7	36·5	39·2	—
1949 stem	—	—	11·8	14·5	18·9	—
Total new growth	0·8	8·5	33·5	51·0	58·1	—
Old needles	26·8	30·9	35·1	25·8	7·8	16·0
Old stem	15·1	20·9	27·8	29·0	28·0	Not sig. on log. scale
Root	8·7	11·5	13·1	14·4	15·3	Not sig. on log. scale
Whole plant	51·4	71·8	109·5	120·2	109·2	—
Log. dry weight of 10 plants						
New growth	0·88	1·91	2·51	2·70	2·76	0·19
Whole plant	2·70	2·84	3·01	3·08	3·03	0·18
Relative growth-rate, per cent. per day		0·83	0·96	0·38	−0·27	

The Ridge plants (Table VII) grew at a rate equalling or exceeding that of plants of the same age in Experiment II, until the beginning of August. There was then a rapid fall of the older needles which is reflected in their loss in weight between August 9 and September 20. During this period the total weight per plant also fell, and even if the weight of fallen needles, say 18 g., is added to the total plant weight on September 20, raising it to 127 g., it will be seen that very little dry-weight increase occurred in August and September. The main gains in weight at this time were in the current year's needles and stem, but even these were small and not significant in contrast to the gains in Experiments I and II during the same period of the year.

In the Furrow plants (Table VIII), there was a steady fall of old needles throughout the summer, and increase in the dry weight of the plant was very small and restricted to the period May 25 to August 3. The absence of weight increase between April 1 and May 25 was probably due to a considerable extent to the low mean temperature of April 1950, viz. 5° C. The period of bud-break, which was shown in Experiments I and II to be one of negligible gain or even loss in weight, was correspondingly prolonged. The April mean temperatures in Experiments I and II were 8° C. and 10° C. respectively.

TABLE VIII

Experiment IV, 1950. Seasonal Changes in Dry Weight and Relative Growth-rate of Plants in their Fifth Year of Growth

	Means of 40 plants				
	Apr. 1	May 25	Aug. 3	Sept. 23	Sig. diff. ($P = 0·05$)
Dry weight per plant, g.					
1950 needles	—	—	7·1	8·2	—
1950 stem	—	—	2·4	2·7	—
Total new growth	—	1·4	9·5	10·9	—
Old needles	8·3	6·7	4·9	2·1	2·3
Old stem	9·4	10·2	10·7	10·9	Not sig. on log. scale
Root	2·6	2·4	2·4	2·8	Not sig. on log. scale
Whole plant	20·3	20·7	27·5	26·7	—
Log. dry weight per plant					
New growth	—	0·085	0·906	1·004	0·198
Whole plant	1·28	1·28	1·39	1·40	Not sign.
Whole plant, means of two occasions	1·280		1·395		0·110
Relative growth-rate, per cent. per day		0	0·36	0·05	

In both these experiments on a heath soil there seem to have been operating factors which markedly restricted growth in late summer compared with Experiments I and II, although even in Experiment I it was possible to show a significant reduction in relative growth-rate after the end of July. No clue to the cause of the difference in late summer growth between these two contrasted pairs of experiments can be found in the records of air temperature, rainfall, or evaporation, and the explanation possibly lies in the soil condition of the heath.

The very poor growth of the Furrow plants was almost certainly due to the compaction of the B horizons of the soil, rather than to any exceptional lack of nutrients. For when this soil was crushed and broken and used in a pot experiment in comparison with other soils, it proved to be quite fertile and to have a considerable reserve of nitrogen. This pot experiment will be described in a later paper.

In contrast to Experiments I and II, in which the weight of roots and old stem showed little increase until the middle of the season, these parts of the plants appeared to increase steadily through Experiments III and IV. The errors of these weights were, however, so high that it was not possible to show in either experiment that the increase was significant. It is doubtful therefore whether much importance should be placed on this particular difference between the two sets of experiments. These heathland experiments were the first ones carried out in this series and they demonstrated the need for higher replication and greater uniformity if precise results were to be obtained.

The mean relative growth-rate in Experiment III from April 8 to September 20 was 0·46 per cent. per day, a figure almost identical with that for plants of the same age in Experiment II. Evidently the loss in weight in August and September has been compensated by more rapid growth earlier in the year. In Experiment IV, growth from April 1 to September 23 was at an average rate of only 0·23 per cent. per day.

4. *Growth in weight during the winter months* (Experiments II and V)

It has already been shown that between October 9, 1951 and April 16, 1952, in Experiment II, there was a significant increase in plant dry weight of approximately 50 per cent., with corresponding increases in the weights of foliage, stem, and root (Table III). In order further to investigate the extent of winter growth in weight, Experiment V was carried out in the winter of 1952–3. Four-year-old plants growing in the same soil as Experiment I were used, and 6 replicates of 6 plants each were taken on four occasions between October 8 and March 30. Results are presented in Table IX. In this experiment the dry weight of the whole plant increased by about 32 per cent. during the 6 winter months, and the buds, foliage, stem, and root all showed individual increases of the same order. By careful size classification at the beginning of the experiment the errors were reduced to a lower level than in any previous experiment, but nevertheless the significant differences are large compared with the increases in weight between any two successive samples. Consequently it is not possible to draw any conclusions about differences in growth-rate at different times of the winter, but growth appears to have proceeded fairly uniformly throughout the experiment, with a mean relative growth-rate of 0·15 per cent. per day.

TABLE IX

Experiment V (1952–3). The Changes in Dry Weight and Relative Growth-rate during the Winter Months of Plants at the End of their Fourth Year of Growth

	Means of 36 plants				Sig. diff.
	Oct. 8	Dec. 1	Feb. 9	Mar. 30	(*P* = 0·05)
Dry weight per plant, g.					
Apical buds . . .	0·364	0·399	0·499	0·496	0·112
Needles	28·8	32·3	34·1	36·5	4·0
Stem and other buds . .	19·8	21·4	23·1	25·8	2·9
Root	18·7	21·1	23·0	26·4	3·5
Whole plant . . .	67·7	75·2	80·7	89·2	—
Log. whole plant . .	1·888	1·933	1·970	1·999	0·043
Relative growth-rate, per cent. per day		0·20	0·12	0·14	

5. *Changes in needle weight and starch content* (Experiments I, II, V, and VI)

The data presented in the foregoing tables show that after the needles have ceased to grow in length or breadth, the dry weight of the old foliage may fluctuate considerably. Some of the losses of dry weight shown in the tables were undoubtedly due to needle-fall, but, as will now be shown, the individual needles may also gain or lose weight. In Experiments I, II, and V the changes of weight per unit needle volume were followed, and the information gained in this way was supplemented by a further experiment, No. VI. In this experiment there were 4 replicates of 10 plants each, in their fifth year of growth. At intervals throughout the year October 1952 to September 1953, 2 needles of each age were taken from the main stems of each of the 40 plants, making 80 needles of each age at each sampling. Because the sampling was restricted to the main stems, and the same plants were used each time, the sampling error of the needle size was low, and the weight per needle appeared to give a sufficiently accurate picture of the gains or losses from the leaves which had ceased growth, without recourse to the calculation of weight per unit volume.

In parallel with these studies of weight per needle or per unit volume of needle, examinations for starch were made in sections, and the starch contents were visually estimated and placed in semi-quantitative grades, which have already been described. A supplementary investigation was carried out to see whether there were diurnal fluctuations sufficiently large to cause appreciable error in the study of seasonal changes. Diurnal change was visually detectable but the difference between daily maxima and minima was of the order of about 1 on the scale used. The results now to be discussed were all obtained from needle samples taken before noon. All the information on weight and starch content is illustrated in Fig. 3.

Throughout their first summer of growth the needles gained in dry weight per unit volume (Experiments I and II). At first their starch content rose also, but it fell again in August and September, and by October the needles contained no starch. Gain in weight of the needles continued slowly through the first winter, but they remained free of starch until after midwinter; they were found to contain a little starch in February (Experiment V), and quite large quantities at the beginning of April (Experiments I, II, and VI). In April and May, when rapid extension growth was taking place, the weight and starch content of the needles, now in their second year, fell sharply. During the rest of the summer changes in weight were rather variable. In Experiment II there was a slight recovery of weight in June, followed by a further decrease, while in Experiments I and VI there was a continued rise. But as in the first-year needles, the starch declined and ultimately disappeared. Through the second winter behaviour was similar to the first; there was a fairly steady increase in weight, but the starch content remained low until after February, and then rose rapidly (Experiment V). The needles began their third year, like their second, with a high starch content, and both their dry weight and their starch fell as extension growth began. In the middle of the summer there was a little recovery of starch content, but all the starch disappeared before the needles fell.

The main points which emerge from the consideration of the data of Fig. 3 are that there was an appreciable accumulation of dry weight by the needles during the winter months and a rapid fall when extension growth began, suggesting the storage and remobilization of reserve materials; and that even after the end of the first summer, when growth in volume had ended, the weight changes could not be identified with changes in starch, for there was a long period from September to February when starch remained very low.

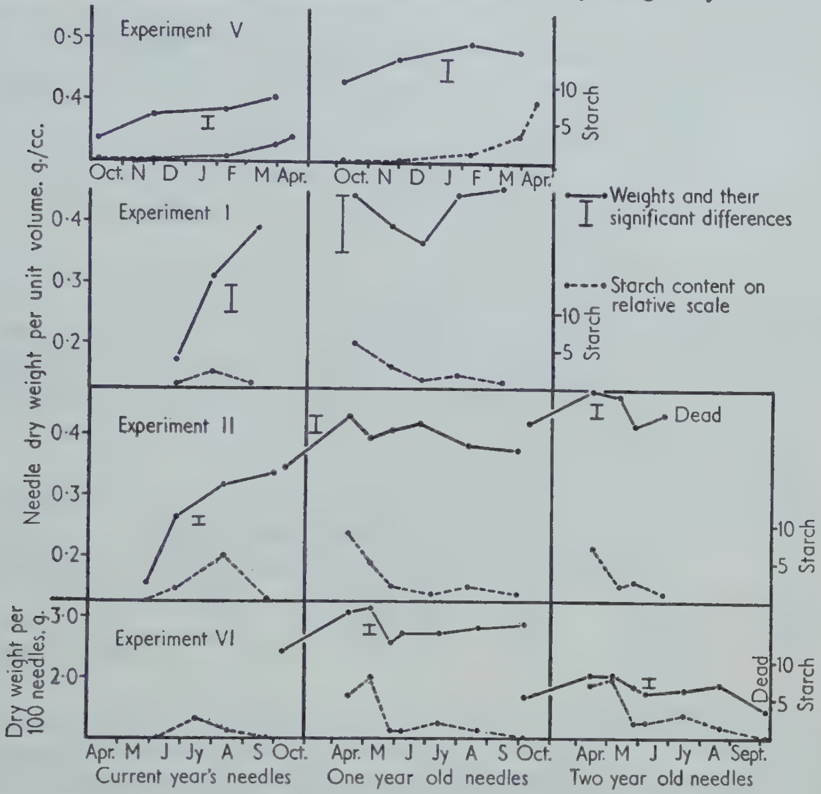

FIG. 3. Seasonal changes in needle dry weight per unit volume (Expts. I, II, and V), or needle dry weight (Expt. VI), and starch content.

TABLE X

Experiment II, 1952. Seasonal Fluctuation in Starch Content of the Stem

Starch content on relative scale, 0–10

	Apr. 17	May 6	May 27	June 26	Aug. 10	Sept. 29
1952 stem	—	—	< 1	2·5	3·5	2
1951 stem	8	7·5	3·5	2·5	5	3

Only in Experiment II were stems examined for starch. The results, given in Table X, showed general agreement with those of Wight (1933), who made a detailed investigation of the starch content of stem and root, mainly with much older trees than these. Excluding the current year's extension, starch was high in April and early May, fell during late May and June to a low level, recovered somewhat in August, and fell again in September.

6. *Changes in net assimilation rate with time of year and age of plant.*

In all the foregoing experiments it has been possible to calculate net assimilation rates by the formula

$$\text{N.A.R.} = \frac{(W_2-W_1)(\log_e L_2-\log_e L_1)}{L_2-L_1}\,\frac{1}{t_2-t_1},$$

where W_1 and W_2 are the dry weights of the plant, and L_1 and L_2 are the dry weights or areas of the foliage, at times t_1 and t_2 respectively.

Williams (1946) has pointed out that the use of this formula may lead to error unless the relation between W and L is linear; and that if it is curved, the error increases with the time interval employed. It has already been shown that in the beginning of the summer the increase of needle weight, and even more of needle area, is more rapid than the increase in weight of the whole plant, and in view of the resulting curvature of the needle-area/plant-weight relation it seemed desirable to assess the error introduced by the use of periods of 4 to 6 weeks in the calculation of N.A.R. In Experiment I the procedure suggested by Williams was followed. This is to obtain a series of weights for shorter intervals than were actually employed in sampling, by interpolation on a smoothed curve of plant weight on time; and to obtain the corresponding needle areas from the smoothed curve of needle area on plant weight. The N.A.R. calculated between two sampling occasions is then compared with the mean of the separate values for the shorter intervals into which the full period has been divided by interpolation.

TABLE XI

Experiment I. Mean Net Assimilation Rates per Unit Needle Area (g./dm.^{2}/week) Calculated over Different Time Intervals

	Apr. 26 to May 25		May 25 to July 30		July 30 to Sept. 11	
Time interval (days) .	10	30	9	35	11	43
N.A.R. . .	0·195	0·200	0·417	0·437	0·216	0·221

The durations of the three periods between samples were 30, 35, and 43 days and they were divided into shorter intervals of 10, 9, and 11 days respectively. The results of the calculations are given in Table XI, from which it appears that the errors caused by calculating N.A.R. over periods of 4 to 6 weeks are unimportant.

The discrepancy between the values in Table XI and those in Table XII is due to the fact that the former have been calculated from the mean weights and areas for the experiment, while the latter have been calculated separately for each replicate and averaged after this process.

Examination of the results presented in Table XII reveals that N.A.R. was low during the winter, and that following bud-break there was, as has already been shown, a period when there was a slight but non-significant loss in weight of the plant. During May to September the mean N.A.R. was five to seven times as great as in the winter months if a comparison is drawn between the experiments covering the third year of growth, and about four times as great in the experiments for the fourth year of growth. In making a statistical analysis of the variation during the summer months, the same procedure had to be followed as has already been described for relative growth-rates, to ensure that only independent estimates were analysed. Although there was considerable variation in N.A.R. during the summer months, it was not significant. It may, however, be noted that the low value between June 23 and August 8 in Experiment II corresponded with an unusually dry period—July rainfall was only 11 mm.—while the high value in the corresponding period in Experiment I was obtained under conditions much wetter than normal, for July rainfall was 92 mm. and the soil was probably near field capacity throughout this time. The relation between soil moisture and N.A.R. will be examined in a later paper.

TABLE XII

Seasonal Changes in Net Assimilation Rate in the Third and Fourth Years of Growth

Third year of growth

Experiment	I					II
Period of growth	Apr. 20 to May 26	May 26 to June 25	June 25 to July 30	July 30 to Sept. 11	Mean, May 26 to Sept. 11	Oct. 9 to Apr. 16
N.A.R., g./dm.^{2}/week	—	0·190	0·465	0·277	0·344±0·057	0·064±0·010
N.A.R., g./g./week	−0·025±0·059	0·134	0·317	0·164	0·224±0·037	0·033±0·008

Fourth year of growth

Experiment	II					V
Period of growth	Apr. 16 to May 6	May 6 to June 23	June 23 to Aug. 8	Aug 8 to Sept. 23	Mean, May 6 to Sept. 23	Oct. 8 to Mar. 30
N.A.R., g./dm.^{2}/week	—	0·221	0·113	0·170	0·195±0·021	0·059±0·013
N.A.R., g./g./week	−0·033±0·053	0·108	0·054	0·082	0·095±0·013	0·027±0·006

Further light was shed on the absence of net assimilation between April 20 and May 26 in Experiment I and between April 16 and May 6 in Experiment II by including in Experiment I treatments in which the needles persisting from the previous year were either removed or else enclosed in a wrapping of aluminium foil, on April 20. By May 26 these treatments had caused dry-weight losses in the defoliated plants of 12·8 per cent. and in the plants with darkened needles of 18·0 per cent. Since untreated plants hardly changed in dry weight during this period and their respiration and real assimilation must have been balanced, the respiration loss of plants with darkened needles can also be used to calculate the rate of real assimilation in the control plants.

The value so obtained is 0·120 g./sq. dm./week, a figure less than the summer values of net assimilation but exceeding the winter value. Although an explanation of this temporary high rate of respiration relative to assimilation might be sought in their differential responses to changing environmental

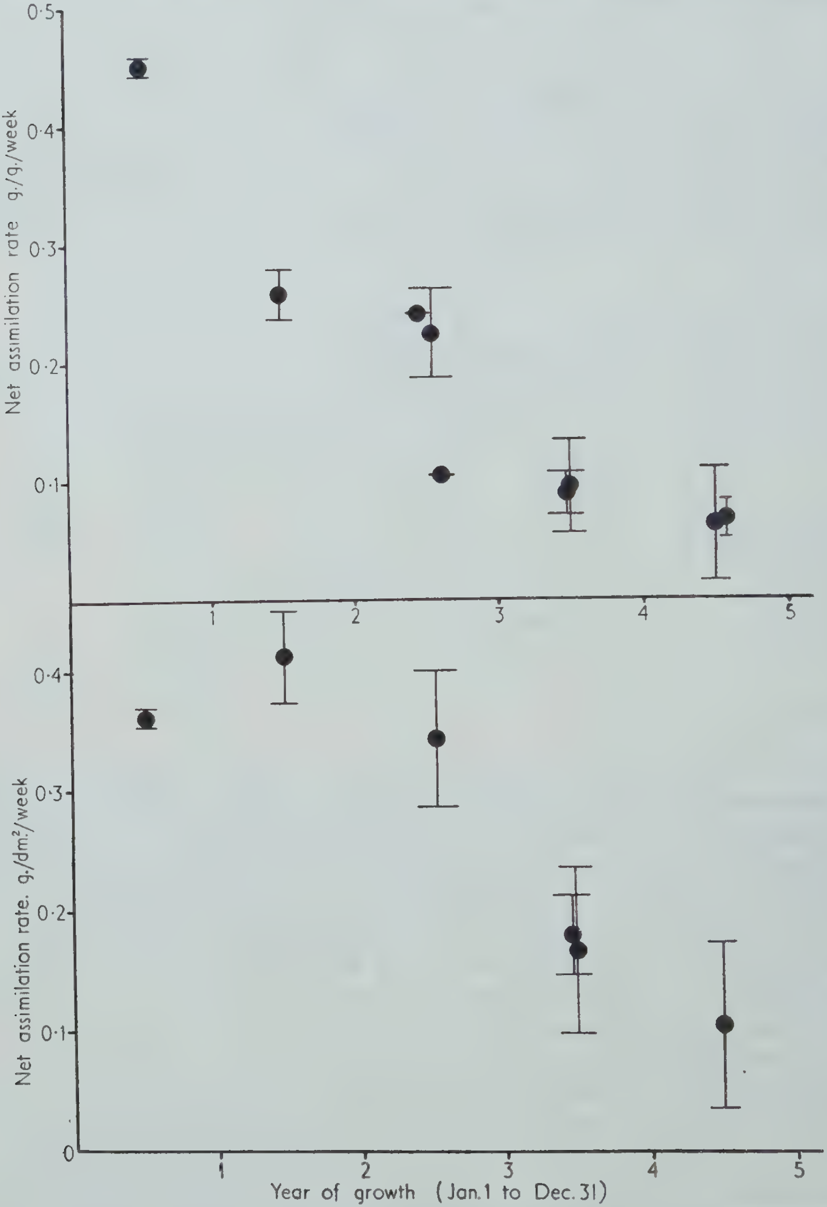

FIG. 4. The variation of net assimilation rate with age of plant. The vertical lines represent standard errors, and the horizontal lines show the period during which the determination was made; see also Table XIII.

factors, especially temperature, it seems most likely that its fundamental cause is ontogenetic.

It will be observed in Table XII that the plants of Experiment I, in their third year, had a higher N.A.R. in summer than the plants of Experiment II, in their fourth year. Data from other experiments were available, covering the first 5 years of growth, and these have been assembled in Fig. 4. In some of the supplementary data needle areas had not been measured, and sampling had been carried out only over restricted periods of the summer. Consequently the data in the figure have been selected to exclude the period October to mid-May, and also periods of heavy needle fall, and more data can be given on a needle-weight than on a needle-area basis. The periods for which these values of N.A.R. have been calculated are given in Table XIII with other data derived from the same experiments.

TABLE XIII

Key to the Net Assimilation Rates in Figure 4, and other Derived Data for the Same Periods of Growth

Year of growth	Experiment no.	Period of growth	Needle wt./plant wt.		New foliage/old foliage at end of period	Relative growth-rate per cent. per day
			Initial	Final		
1		June 10–Aug. 8	0·63	0·64	—	4·27
2		May 23–July 31	0·42	0·63	10·5	2·19
3	I	May 26–Sept. 11	0·35	0·51	6·0	1·33
3	*	May 22–July 9	0·63	0·62	*	2·28
		July 9–Sept. 11	0·62	0·58	*	1·29
4	II	May 6–Aug. 8	0·38	0·48	3·1	0·56
4	III	May 17–Aug. 9	0·43	0·52	1·4	0·62
5	IV	May 25–Aug. 3	0·32	0·44	1·4	0·35
5		July 10–Aug. 13	0·38	0·39	0·8	0·33

* From data of Bauer (1910). Old needles were few relative to new, and many of them were shed early in the year.

Although the data have been obtained from experiments carried out in different years and under different conditions of nutrition and water-supply, they provide clear evidence of a fall in N.A.R. with age. The main effect of expressing the values on a basis of needle area rather than needle weight is to eliminate an apparent marked drop in assimilation rate between the 1-year-old plants, whose foliage was of the juvenile form, and the 2- and 3-year-old plants, whose foliage almost entirely consisted of adult type needles. If the 1-year-old plants are excluded the proportionate fall of N.A.R. with age is much the same whether expressed on a basis of needle weight or needle area. There is some suggestion in the shape of the curves that at 5 years they are approaching a minimum.

In a search for an explanation of the falling N.A.R., two other characteristics of the plants have been calculated and included in Table XIII. The ratio of the area or weight of needles to the weight of the whole plant, which is frequently calculated because of its influence on relative growth-rate, may also be of significance because it shows the relative amounts of assimilating and respiring material; and any alteration in this ratio may affect the magnitude of net assimilation. Since needle areas are not available for all the experiments in Table XIII, only needle-weight ratios are shown. The ratio of the current year's needles to older needles may also be of significance if the assimilation rate of the needles falls with age. Table XIII shows that both the needle-weight ratio and the proportion of new to old needles declines with age. The significance of these observations will be discussed below.

Since both N.A.R. and needle weight or area ratio decline with age, relative growth-rate will also do so, and the magnitude of the fall is illustrated in Table XIII, where the relative growth-rates have been calculated over the same periods as the N.A.R.s.

DISCUSSION

The information which has been obtained on the annual cycle of weight change in *P. sylvestris* may now be discussed in relation to previous work.

That the plants should assimilate in the relatively mild winters of southern England is not unexpected, for Zacharova (1929) found that *P. sylvestris* could carry on assimilation at temperatures at least as low as −9° C. near Moscow, and Freeland (1944) obtained a similar result at Lake Michigan. In two experiments in the present series, plants have been found to increase in dry weight during the winter by an amount approximately equal to the dry weight of the needles at the beginning of the period, but in view of the fall with age which takes place in the summer rate of net assimilation, a similar fall may occur in the winter rates.

At the beginning of April there occurs a short period in which there is no change in the weight of the plant, a period whose beginning and end are defined approximately by the first signs of bud growth, and the first extension of the new needles beyond their subtending scales. Something comparable with this temporary cessation of net assimilation is also to be seen in the results of the two investigations cited in the last paragraph. The plants used by Zacharova were enclosed in glass chambers on her laboratory roof, and on sunny days the air temperature around the plants rose above that outside. Air temperature in the chambers was about −9° C. in early February, but rose to +12·3 and +22·7° C. on March 9 and 21 respectively. With these high temperatures, carbon dioxide uptake, which had been positive throughout January and February, was transformed to loss. In Freeland's experiments also, while assimilation was noted in January, February, and early March at temperatures down to −9° C., on March 31, when the temperature rose to 24° C., and light intensity was normal, there was no uptake of carbon dioxide. Both these investigations therefore show evidence of a period when respiration equalled or exceeded photosynthesis at the end of the winter, but it is uncertain whether this corresponded to the period of low or negative net assimilation which has been found in the present work to occur at bud-break, for no information is given by either Zacharova or Freeland on when their plants began active growth. Both ended their experiments when temperatures during the day rose above 20° C., and neither comment on the lack of apparent assimilation in these conditions.

It should not be supposed that this rise of respiration over photosynthesis is to be simply explained by a differential response of these two processes to rising temperature, for there are internal changes taking place in the plant during the winter months which are also likely to affect the relationship between photosynthesis and respiration. Wareing (1951) has shown that *P. sylvestris*, like many other perennial plants of temperate climates, passes through a period of physiological, or internally caused, dormancy in winter, when it is incapable of growth (cell division or extension) even in favourable external conditions. The ending of this dormancy is hastened by the natural low-temperature conditions of winter, and Wareing showed that the buds became capable of renewed growth at least by the beginning of March. The change from measurable net assimilation in February and March to weight standstill or loss in April must then be considered in conjunction with other physiological changes accompanying the ending of dormancy.

During the period of bud extension when photosynthesis is only sufficient to balance respiration, the old needles show a significant loss in weight, and the fact that this approximately equals the gain in weight by the extending shoots suggests that their initial growth is mainly at the expense of materials stored in the old needles during the winter. It would, however, be unjustified to assume that the extending shoots cannot also draw for their growth on reserves in the stem and root, for in the plants of Experiment I, referred to in the section on Net Assimilation Rate, whose needles were removed on April 20, the buds grew normally during the following month although their dry weight gain by May 26 was about 30 per cent. less than that shown by control plants.

Wight (1933) reported that cambial activity begins before the buds break, and Wareing (1951) observed that by May 1 (in southern England) quite a wide band of wood had been formed in 2-year-old plants. Root extension has also been observed to begin before bud-break and both Wight (1933) working with *P. sylvestris* and Kienholz (1934) with *P. strobus* found that the period of rapid bud growth is also one of rapid root elongation. It will be seen therefore that there is rapid cell growth taking place in all parts of the plant at a time when net assimilation is negligible, and the assimilates accumulated during the winter months are no doubt of considerable importance at this time.

The course of growth during the summer period of relatively rapid weight increase has been described and discussed in connexion with Tables IV and VI, and Fig. 2, which show how the young needles are rapidly expanded in the first part of the summer and that after the end of June the dry matter assimilated is increasingly diverted to the woody stem and roots.

Although the estimates of N.A.R. are not sufficiently accurate for the detection of any but the largest differences in a particular year, they show a clear fall from year to year as the plant ages, and the possible causes of this will now be considered. The large difference in N.A.R. per unit weight between 1-year-old and 2-year-old plants disappears when the values are transformed to a leaf-area basis, because of the difference of the ratio of area to weight between the juvenile and adult form of the needles. But the foliage of 2-year-old and older plants consists almost entirely of needles of adult form, and although the relations between weight, area, and volume of needles may vary with environmental factors, there is no evidence that they change with the age of the plant, and the N.A.R. declines with age, whatever basis is used for its expression.

However, it is shown in Table XIII that as the plant ages the proportion of current year's to older needles falls, and it might be thought that the fall of N.A.R. could be due to the increasing proportion of old needles, if their photosynthetic rate fell with age.

Stålfelt (1924) compared the apparent assimilation rate of needles of different age in *P. sylvestris*, and although his results varied in detail according to the light intensity in which the experiments were carried out, and the method of expressing assimilation, they pointed to a slight rise in assimilation rate with age. On the other hand, Freeland (1952) in similar experiments carried out in a light intensity of 2,200 foot-candles found the assimilation rate of first-, second-, and third-year needles to be 1·4, 1·1, and 1·0 mg. carbon dioxide/100 leaves/hour respectively. While Freeland's results suggest that ageing of the needles may contribute to the fall of N.A.R., they would only account for a fall of 12 per cent. between the 2-year-old and 5-year-old plants, whereas a drop of about 75 per cent. is in fact found.

Another factor to be considered is that as the plant ages its needle-area ratio or needle-weight ratio falls, i.e. the proportion of photosynthesizing tissue to respiring tissue decreases. From Polster's (1950) extensive observations on the gas exchange of *P. sylvestris* it appears that a loss of 10 per cent. of the needle dry weight per week is a not unreasonable estimate of respiration in the summer months. But even if it could be assumed that all the tissues of the plant respired at this rate it would only be sufficient to reduce the N.A.R. from 0·25 g./g./week in 2-year-old plants with a needle-weight ratio of 0·5 to 0·20 g./g./week in 5-year-old plants whose needle-weight ratio is about 0·4 (Table XIII). A more marked fall would of course occur if the increase in age of the plant were accompanied by an increased proportion of tissues of high respiration rate in the non-photosynthetic part of the plant, or by an accelerated rate of decay of root tissues. Appreciable loss in weight by needle fall did not contribute to the data of Fig. 4 here considered.

A further factor whose importance in these experiments cannot be estimated quantitatively is the increase in mutual shading by the needles. In 1-, 2-, and even 3-year-old plants most of the foliage consists of the current year's needles which, produced at the periphery of the crown space of the plant, receive the maximum possible illumination. But in older plants where 1-year-old and 2-year-old needles form an appreciable part of the total foliage, the shading of these older needles by the current year's shoots may be important. Stålfelt (1924) found that if needles were removed from a shoot of *Picea excelsa* to leave only two opposite rows which did not shade each other, the apparent assimilation rate of the remaining needles was raised appreciably, and Uhl (1937) showed that differences between the assimilation rates of different species of *Pinus* were closely related to the density of arrangement of the needles on the twig and the degree of mutual shading. But as with other possible causes which have been discussed, an increase in mutual shading seems inadequate to explain more than a small part of the fall in N.A.R. For if it were an important factor, then needles on the fully illuminated parts of a tree, or removed from the tree and exposed to full light, should not show low assimilation rates. The available evidence is complicated by the fact that N.A.R.s determined by growth analysis methods cannot be readily compared with the apparent assimilation of carbon dioxide by the needles alone, but it may be pointed out that while the N.A.R. of 1- to 2-year-old plants of *P. sylvestris*, say 0·35–0·40 g./dm.^{2}/week, is little less than the mean value of about 0·55 g./dm.^{2}/week reported by Heath and Gregory (1938) for a diverse range of mesophytes, there is ample evidence that even fully illuminated shoots on older trees of this species have a low rate of apparent assimilation. For instance, Stålfelt (1924), Iwanoff and Kossowitsch (1929), Uhl (1937), and Polster (1950) all obtained values of the order of 1·5 to 3·0 mg. CO_2/g. fresh wt./hour—values which must be multiplied by a factor of between 2 and 3 to convert them to a leaf dry weight basis—or of 5 mg./dm.^{2}/hour. In contrast Verduin (1953) gives mean values for photosynthetic rates of temperate zone herbaceous mesophytes in natural conditions which are equivalent to 44 mg./CO_2/g. dry wt./hour or 18 mg./dm.^{2}/hour, and which must be reduced by about 1/16th to allow for respiration.

Thus it appears that the low N.A.R. of the 5-year-old plants is to be explained by a low rate of apparent assimilation even in fully illuminated shoots rather than by increased respiration or other form of dry-weight loss in the non-photosynthetic tissues of the plant; and the conclusion is reached that the photosynthetic or respiratory activity of the needles is in some way conditioned by the age of the plant on which they are borne.

In view of the marked fall in N.A.R. during the first 5 years of growth it would be interesting to know for how long, or how rapidly, this fall continues. Ovington (1957) has calculated the total production of dry matter/m.^{2} leaf surface/annum in stands of different ages. Between the third and seventh years he obtained a value of 0·18 kg. The rate of production then showed a certain decline, which Ovington ascribes to canopy closure and intense competition in the young, unthinned stands, for with the first thinning at 20 years, the rate of production rose sharply and from 23 to 35 years the values were between 0·20 and 0·25 kg./m.^{2} leaf surface/annum, and the subsequent apparent fall may have been due to the fact that the 55-year sample was of poorer growth quality than the preceding ones. Despite some variability, Ovington's data give little evidence of any systematic fall of N.A.R. with age from 3 to 35 years, and the average rate of production over the whole period was 0·18 mg./m.^{2} leaf surface/annum. Burger (1948) also showed that the dry-weight increment of timber per unit needle weight varied little with age in *P. sylvestris* stands varying from 20 to 100 years old.

An attempt has been made to compare the N.A.R.s obtained in the present paper with Ovington's value for production. Some allowance has to be made for the annual fluctuation in leaf area. Ovington measured leaf areas in mid-August, by which time the greater part of the third course of needles has been shed. The leaf area measured in the autumn will not vary much through the winter, but in the following summer a new course of needles will be expanded before much shedding of the old occurs. About midsummer the leaf area will in consequence be 50 per cent. greater than in the previous autumn, and perhaps 25 per cent. greater on average over the whole of the summer months.

Now the N.A.R. found for the winter months at the end of the fourth year of growth was 0·06 g./dm.^{2}/week so that 1 dm.^{2} needles in 26 weeks in winter would produce 1·56 g. dry matter. Allowing for a short period when net assimilation is zero, there would be a summer period of say 20 weeks when the equivalent needle area would be 1·25 dm.^{2}, and the N.A.R. 0·10 g./dm.^{2}/week; this was the lowest value obtained, in the fifth summer of growth (Fig. 4). The corresponding dry-weight production would be 2·50 g., and the total dry-weight production in the year for an initial leaf area of 1 dm.^{2} would be 4·06 g. Now the areas used in this calculation are simply the product of length and breadth, and the rate of production has to be divided by 2·57 in order to express it on the basis of superficial area used by Ovington. After making this transformation, and changing the units of weight and area, an estimate of production of 0·16 kg./m.^{2} leaf surface in autumn/annum is obtained, which agrees closely with the value found by Ovington from the fourth to the seventh years inclusive and also as a mean throughout the 55 years covered by his stands.

The evidence seems conclusive that even in summer *P. sylvestris* has a N.A.R. which is probably lower than any yet recorded for plants growing in natural and unshaded conditions, and yet, as Ovington has shown, the total dry-matter production of the stands in which he worked was considerably greater than that of arable crops and hay in the same neighbourhood. As he, and also Pearsall (1954), have pointed out, the explanation is to be sought in the evergreen habit, which as a complement to the determinations of N.A.R. may be given quantitative expression in the terms introduced by Watson (1947). The average needle surface area found by Ovington (1957) in stands of 20 years and older was 8·5 Ha./Ha. land surface. Dividing this by 2·57 to make it comparable with the area component of N.A.R., a Leaf Area Index of 3·3 is obtained. Similarly L.A.I. values of about 2·9 may be obtained from the data published by Burger (1941, 1948) for a number of stands in Switzerland ranging in age from 32 to 88 years. Multiplying these values by 52 weeks, the Leaf Area Duration is at least 150 weeks, compared with values of 17 to 33 weeks which were found by Watson for various annual crops.

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Experimental and Analytical Studies of Pteridophytes

XXXVII. A Note on the Inception of Microphylls and Macrophylls

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With Plate XVI and fourteen Figures in the Text

ABSTRACT

In an attempt to shed new light on the nature of microphylls and macrophylls, a study has been made of leaf inception in selected materials, including *Psilotum*, *Tmesipteris* and other pteridophytes, and flowering plants such as *Cuscuta* with greatly 'reduced' leaves. It is shown that the incipient primordia of the small scale-like leaves of *Psilotum* and of the quite substantial, though microphyllous, laminate leaves of *Tmesipteris* are closely comparable and that there are no essential differences in the histological organization of incipient microphylls and incipient macrophylls. In parasitic species such as *Cuscuta*, with small scale-like leaves, the organization of the apical meristem and the inception of primordia are as in normal autotrophic species. The so-called 'reduced' leaves and the microphyllous condition of some pteridophytes are attributable to a physiological-genetical limitation of growth of primordia in the subapical regions of the shoot. The conclusions which may be drawn from these observations are discussed.

INTRODUCTION

THE *microphylls* of the Psilophytineae, Equisetineae and Lycopodineae and the *macrophylls* of the Filicineae and Spermatophyta have been considered by some botanists to be quite distinct organs both in their organization and evolutionary origin. Microphylls are usually, but not invariably, small; the lamina has typically a single unbranched vascular strand which, in some instances, is reduced or absent. Macrophylls are usually of considerably larger size, though exceptions are known; they are characterized by a lamina with many veins. The controversial aspects of the evolutionary origin and of the nature of these contrasting foliar members have been given detailed treatment in standard works on morphology (e.g. Bower, 1935). Readers may, however, be reminded that the macrophyllous leaf, as in the ferns, is thought to have been evolved by the progressive modification of a lateral dichotomous branch system, whereas microphylls have been regarded as tentative upgrade steps from leaflessness. Bower, for example, described microphylls as enations, by which he meant 'lateral outgrowths of a surface previously untenanted'. Some botanists, however, consider that all microphylls are reduced forms of macrophylls (e.g. Zimmermann, 1930). Microphylls have also been regarded as small leaves that do not differ in essentials from macrophylls. The present investigation was undertaken because it seemed to the writer that new light might be shed on this problem by a study of leaf inception in selected materials.

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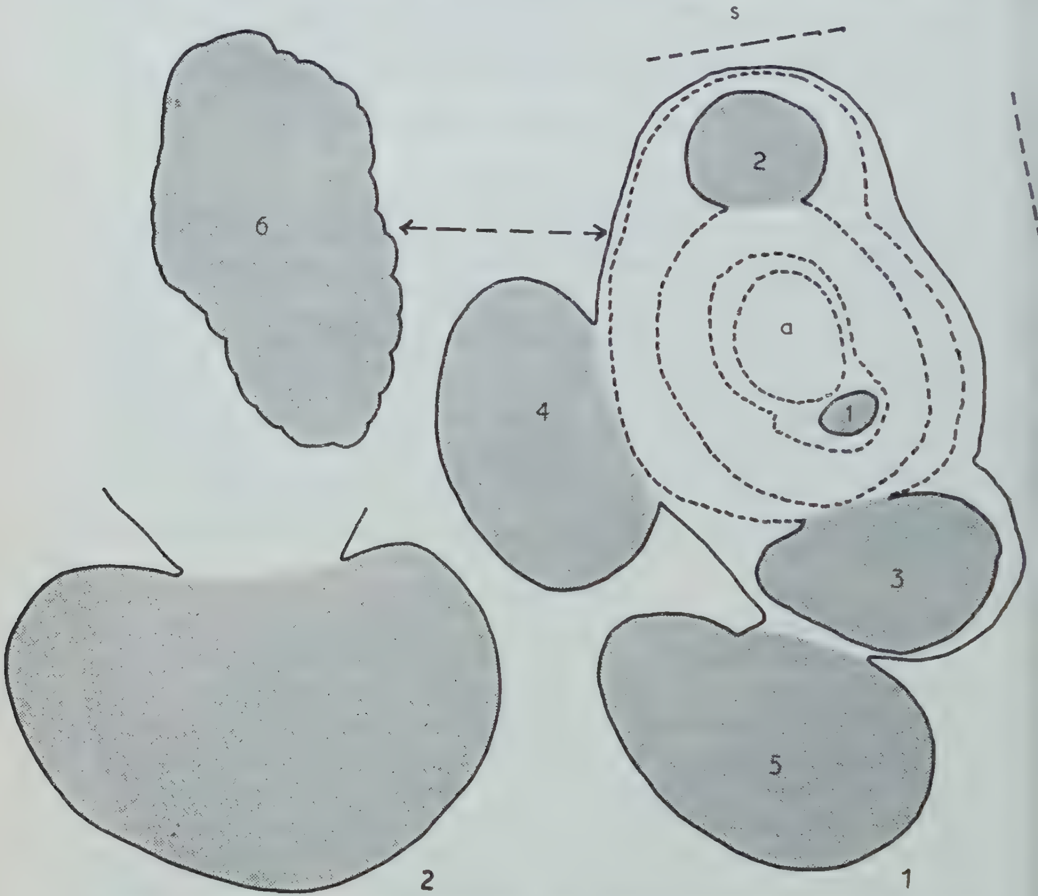
LEAF FORMATION IN PSILOTUM AND TMESIPTERIS

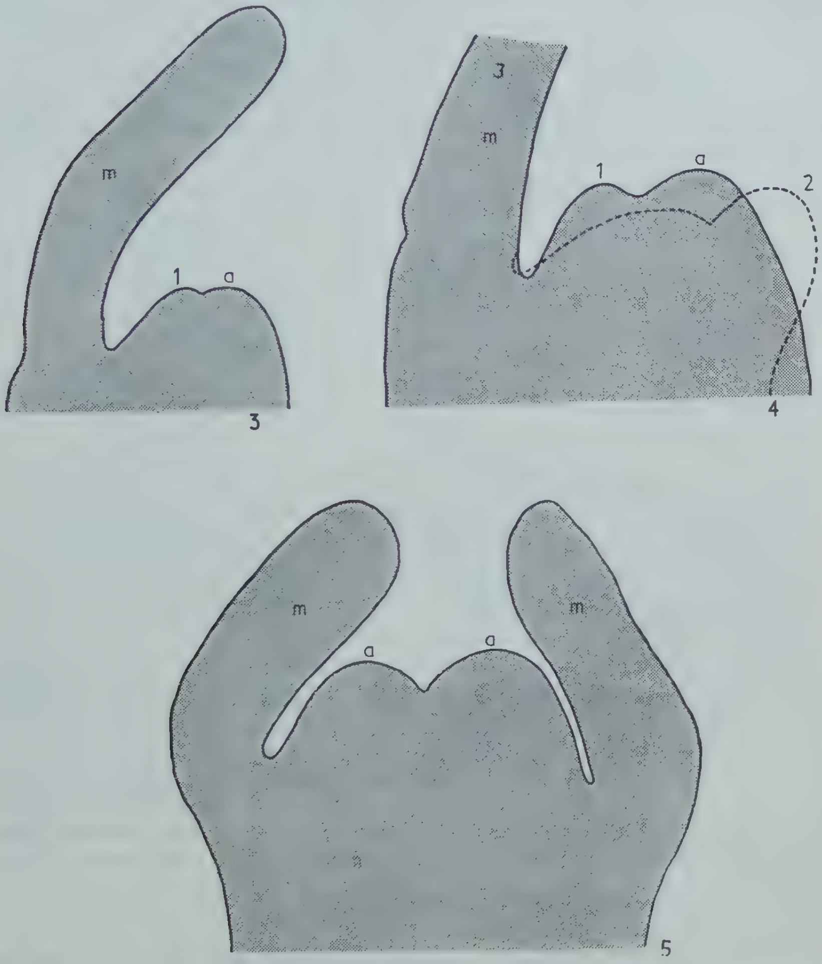
Microphylls of virtually the smallest and simplest type are present on the aerial shoots of *Psilotum*; they are, indeed, little more than small spiny scales. In *P. nudum* (*P. triquetrum*) they consist of enlarged parenchymatous cells and are devoid of stomata and vascular tissue: in *P. flaccidum* a small vascular strand runs between the base of the microphyll and the shoot stele. The inception of such a small adult lateral member at the shoot apex is clearly a matter of some interest. The essential facts as ascertained from serial transverse and longitudinal sections of *P. nudum* are illustrated in Text-figs. 1–6, and Pl. XVI, Figs. 1 and 2. As in all other vascular plants, the primordia originate in the shoot apical meristem. It will be noted that, at its inception,



TEXT-FIGS. 1, 2. *Psilotum nudum* (*P. triquetrum*). Fig. 1. Superimposed serial transverse sections of the apex of a vegetative shoot, showing the shoot apex (*a*), leaf primordia 1–6, and the sutures (*s*) of two dichotomies.

Fig. 2. Cross-section of a fully formed microphyll: it consists of large parenchyma cells and there is no vascular strand. (× 100.)

a primordium is of quite large size relative to the apical meristem, and that it comprises both superficial and sub-surface cells. The prism-shaped surface cells elongate radially and some of them can be seen to be dividing, or to have divided recently, by periclinal walls. The underlying cells afford evidence of active division in different directions. In short, the microphyll in *Psilotum* appears to originate in quite a large growth centre. These histological developments, moreover, closely resemble those of the macrophyll in a leptosporangiate fern (Text-fig. 7, and Pl. XVI, Fig. 4; and of seed plants, Pl. XVI, Fig. 3). Unlike a fern primordium, however, in which the apex remains actively meri-

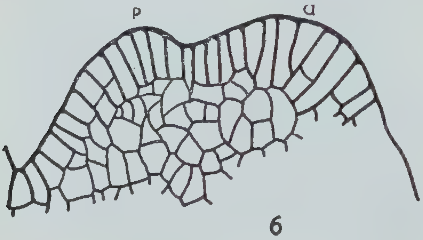


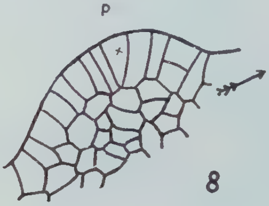
TEXT-FIGS. 3–5. *Psilotum nudum.* Fig. 3. Longitudinal median section of the last-formed leaf primordium (1) and of the shoot apex (*a*); *m*, fully formed microphyll.

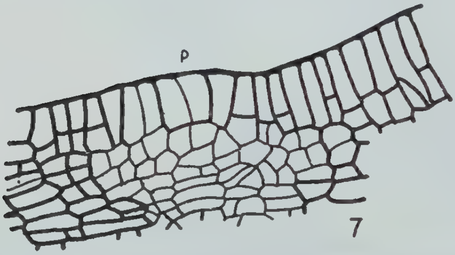
Fig. 4, as in Fig. 3. The size and position of primordium *2* and of primordium *3* are also shown.

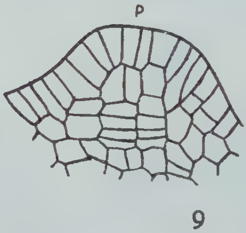
Fig. 5. An apical dichotomy with two microphylls in characteristic positions. (×67.)

stematic for a considerable period, the meristematic activity of the leaf primordium in *Psilotum* is short-lived. After some further cell division, the subsequent development consists entirely of an early and conspicuous enlargement of the individual cells. As a result, the microphyll is fully developed while still close to the shoot apex and is of large size relative to it. However, as there is no further leaf development during the subsequent considerable enlargement of the axis, the leaves on the mature stem appear small and insignificant. The two small microphylls associated with each synangium (the sporogenous member) follow a similar course of development. Bierhorst (1954) has given an account of the relation of the microphylls to shoot dichotomy.









TEXT-FIG. 6. *Psilotum nudum.* Longitudinal median section of a young leaf primordium (*p*) and of the shoot apex (*a*); in the multicellular primordium there are periclinal divisions in the surface cells and somewhat irregular divisions in the cells lying within.

TEXT-FIG. 7. *Dryopteris aristata.* Longitudinal median section of a very young leaf primordium (*p*), both surface and sub-surface cells being involved in its formation; shoot apex to right.

TEXT-FIGS. 8, 9. *Tmesipteris tannensis* (redrawn from Bower). Fig. 8. Young leaf primordium (*p*) as seen in longitudinal median section, with apical cell; the arrow indicates the direction of the shoot apex.

Fig. 9. Leaf primordium as seen in a transverse section of the apex. (All × 112.)

Tmesipteris, the other genus of living Psilophytineae (Order Psilotales) is also microphyllous in that its leaves have only a single central vein. These leaves, however, are functional photosynthetic organs and are of quite large size both actually and relatively to the shoot. This fact, indeed, proved not a little troublesome to botanists of a past age in that the two genera are evidently of close taxonomic affinity as judged by their life-history, gametophyte, embryogeny, rootless condition, vascular anatomy, and sporogenous members. Like *Psilotum*, *Tmesipteris* consists of an underground, rootless, branching, rhizomatous region which gives rise to an occasionally bifurcating aerial shoot. The basal region of an aerial shoot is quite devoid of foliar members, but, a little higher up, small spiny outgrowths, like the microphylls of *Psilotum*, are present. On proceeding up the shoot there is a gradual increase in the size of these lateral members so that the major part of the axis is clothed with conspicuous foliage leaves. These are about half an inch long, each with a single vein and a small distal tip or apiculus. In a well-developed specimen, this foliar region is continued into the fertile region where synangia are associated with rather smaller bifurcating foliar organs. Material of critical importance in the present investigation would have consisted of partly grown stout leafy shoots which had not yet entered on the sporogenous phase. Unfortunately, such material was not available. However, an adequate account of just such material has been given by Bower (1894). The shoot apex in *Tmesipteris* is closely comparable with that of *Psilotum*, the superficial layer of the meristem consisting of large prismatic cells, somewhat irregular in size and arrangement. When a leaf is being formed, a group of these prism-shaped cells yield evidence of enlargement and division by periclinal and anticlinal walls and there is also active division in the underlying cells (Text-figs. 8 and 9). The result is a somewhat bulky multicellular outgrowth, closely comparable with the primordium of *Psilotum*. The summit of the primordium in *Tmesipteris*, as seen in a radial longitudinal section, is occupied 'by a single larger cell of a wedge-like or prismatic form' (Bower), and, unlike *Psilotum*, this apex retains its meristematic properties and potentialities. It continues to grow and gives rise to a considerable leaf with a central vascular strand. Eventually, however, the leaf apex ceases to be an active meristematic region: its cells become parenchymatous as in the primordium of *Psilotum*, with the result that a small terminal spine-like outgrowth, sometimes described as a ' mucro' or 'apiculus', is formed. This organ is not unlike the small scale-leaves at the base of the shoot or those of *Psilotum*.

The insignificant microphylls of *Psilotum* and the conspicuous leaves of *Tmesipteris* are thus alike in that their incipient primordia are bulky multicellular outgrowths of the apical meristem and are of quite large size relative to it. The histological details, moreover, are in general accord with those for the inception of leaf primordia in ferns and seed plants (Wardlaw, 1956*a*).[1]

LYCOPODINEAE AND EQUISETINEAE

The species of *Lycopodium* exemplify in a high degree the microphyllous type of organization. So also do other members of the Lycopodineae, though many of the fossil members had leaves of quite large size but usually with a single vein. This is also found in the living genus *Isoetes*. When, however, we examine the inception of a leaf primordium in *Lycopodium* and its relationship to the shoot apex, we find that the histological details are in general accord with those for other vascular plants. Primordia originate on the periphery of a broad, convex meristem and are of small relative size; but they are multicellular from the outset, as is well shown in Strasburger's classical illustration of *L. selago*, and comprise both superficial and sub-surface cells of the meristem. They present, indeed, an appearance which is not unlike that found in certain flowering plants in which primordia of small relative size are formed near the periphery of a large apical meristem. In small plants, or in plantlings formed from gemmae in *L. selago*, the youngest leaf primordia are seen to be of quite large size relative to the apical meristem (Text-fig. 10). *Selaginella* and *Isoetes* yield comparable information. In the embryogeny in all three genera, the first leaves are of quite large size relative to the shoot apex and they may indeed predominate in the early phases of the embryogenesis. The microphylls of *Equisetum*, though they are greatly reduced foliar members, show comparable initial histological developments.

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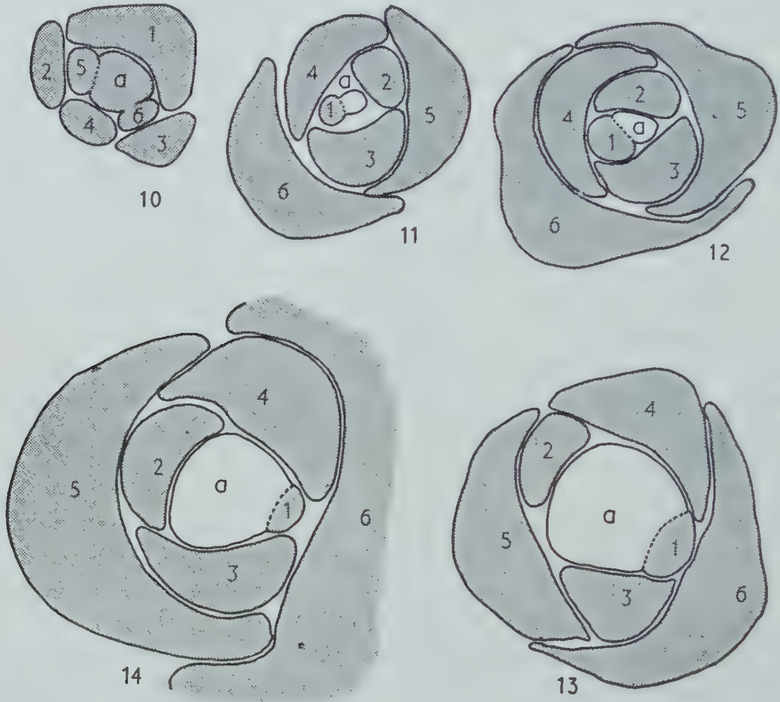
[1] Since going to press, confirmatory evidence has been found in papers by Vetter (1951) and Bierhorst (1956).



Text-figs. 10–14. Various apices, cut in transverse section at or near the axil of the youngest primordium. Except in Fig. 10, where the *oldest* leaf is indicated as 1, the leaves are numbered in the order of their increasing age, i.e. *1*, *2*, &c.

Fig. 10. *Lycopodium selago*, young plant from gemma.

Fig. 11. *Cassytha filiformis*, a parasite with 'reduced' scale leaves on the adult shoot.

Fig. 12. *Rosa multiflora.*

Fig. 13. *Cuscuta americana*, a parasite with greatly 'reduced' scale leaves on the adult shoot.

Fig. 14. *Ranunculus acris.* (All ×67.)

Flowering Plants with 'Reduced' Leaves

Further information of direct relevance to the subject under consideration has been sought in species of flowering plants with 'reduced' leaves, e.g.

parasites and partial parasites. In passing, it may be noted that in autotrophic species with small adult leaves, such as *Hippuris* and *Elodea*, the primordia originate in positions well down on the flanks of the apical meristem and are of small relative size; but, like the incipient primordia of other flowering plants, they comprise cells of the surface and underlying layers of the meristem. Similarly, in the inception of phyllodes, like those of *Acacia* spp. as described by Boke (1940), the histological details are in accord with those for normal foliage leaves.

As examples of leaf inception in species with 'reduced', almost scale-like, leaves, the apices of *Cassytha filiformis* and *Cuscuta americana* are illustrated (Text-figs. 11 and 13). These apices have already been described in some detail by Cutter (1955). Here it will suffice to note that the histological details of leaf inception, and the size of the young primordia relative to the shoot apex, are similar to those in autotrophic dicotyledons. Equivalent sections of the apices of *Rosa multiflora* and *Ranunculus acris* are illustrated for comparison (Text-figs. 12 and 14). Indeed, as Cutter (1955) has shown, no special or unusual features are to be observed in the apices of flowering plant species with irregular nutrition and a concomitant reduction in leaf size. This is also borne out in the embryogeny of such species as have been investigated (Wardlaw, 1955).

Discussion

The essential point that emerges from this investigation is that however much the fully formed leaves of different species may differ, leading to their being assigned to distinct morphological categories such as microphylls and macrophylls, they are all closely comparable at the time of their inception at the shoot apical meristem. In other words, the incipient leaf primordia in all classes of vascular plants show a remarkable homology of organization. Goebel (1900) maintained that all the foliar members in any particular species, no matter how diverse in shape and size they might be, were essentially alike at their inception, the nutritional status of the apex being an important determinant in the subsequent development. The 'juvenile' and 'adult' leaves in ferns, for example, are formed from essentially similar primordia; and this applies also to the small scale-leaves and the large and elaborate laminate leaves of a sycamore or horse-chestnut. The evidence presented here indicates that many of the so-called 'reduced' leaves begin as normal primordia but fail to maintain growth and development beyond a certain stage. Moreover, not all small or microphyllous leaves are necessarily 'reduced' in the phylogenetic sense: as Bower (1935) has said, they may be upgrade developments from an ancestral state of leaflessness.

Psilotum and *Tmesipteris*, with small scale-leaves and relatively large leaves respectively, were selected as affording an opportunity of comparing the inception of primordia in related genera. There is general agreement that the Psilotales are probably the most primitive living vascular plants. As early as 1881 Bertrand, in his comprehensive memoir, had maintained that the leaves of *Psilotum* are greatly reduced structures—'simple emergences on the surface of the plant'—and that they could only be given the morphological value of leaves because of our knowledge of the leafy character of *Tmesipteris*. In a footnote he also refers to the scale-leaves in *Psilotum* as being 'useless rudimentary organs'. Bertrand's view that the scale-leaves of *Psilotum* are derivative rather than primary, and that they have been 'reduced' during descent from an ancestral form with larger leaves, has commended itself to many botanists in the past. The polyploidy and the high chromosome numbers (*P. nudum* (*P. triquetrum*) diploid sporophyte, over 100; tetraploid sporophyte, over 200; *Tmesipteris tannensis*, polyploid sporophyte, between 400 and 500), as ascertained by Manton (1950), may be considered to support this conception; i.e. the species of *Psilotum* and *Tmesipteris* may be the end products of very ancient polyploid series. Comparative morphological studies led Bower to a different view, namely, that the minute scale-leaves of *Psilotum* 'appear to be nascent, and comparable with the scales of *Thursophyton* and other early fossils'. But here the relatively large leaves of *Tmesipteris* presented a difficulty. This Bower attempted to overcome by homologizing the apiculus in *Tmesipteris* with the leaf of *Psilotum*, and by regarding the laterally flattened leaf blade of the former as 'a cladode-like intercalation of indeterminate character'. The present study has shown that the organizational and histological details of leaf inception in *Psilotum* and *Tmesipteris* are closely comparable and are in general agreement with what we know of this phenomenon in all classes of vascular plants. But whereas the incipient primordium in *Tmesipteris* grows into a vascularized photosynthetic leaf, in *Psilotum* it fails to maintain its meristematic character and develops precociously into a parenchymatous scale-like organ. The 'reduced' leaf in *Psilotum*, in brief, is a normal primordium whose further elaboration is precluded by factors in the genetical constitution. Recently, Marsden and Wetmore (1954) have shown that the excised apex of *Psilotum* can be grown in aseptic culture media of known composition and it will be a matter of great interest to see if, by the addition of certain ingredients to the medium, leaf development can be modified in any way.

Investigators such as Lignier (1903) held that microphyllous leaves, as in lycopods, belong to an entirely different category of organs from macrophyllous leaves. More recently Hamshaw Thomas (1950) has expressed the view that, on the evidence from ancient plants, the leaf cannot be regarded as a fundamental unit in plant construction and that leaves may have evolved in several different ways. In an earlier paper he suggested that the difference between a microphyll and a macrophyll may be as great as that between shoot and root (1932). As we have seen, the facts of organogenesis are against this view: in the manner of their inception, there is no essential difference between microphylls and macrophylls though very marked differences become evident during their further development. In the view of Zimmermann (1930), all microphyllous leaves are reduced forms of macrophyllous leaves. Martens (1950) has questioned whether the 'aphyllous' condition in the Rhyniales is truly primitive (see also Wardlaw, 1956*b*).

The general position may now be restated as follows: (i) The organizational and histological aspects of leaf inception are generally comparable in all vascular plants. (ii) As determined by factors in the genetical constitution, the primordia of some species have only a limited potentiality for growth. Some of these belong to the category described by Bower as being on the upgrade from leaflessness; others are indicative of a limitation of a greater potentiality for growth possessed by ancestral forms.

Since there are apparently no essential differences between incipient microphylls and incipient macrophylls, and since there are such very evident differences between them in their adult states, it is important that an attempt should be made to define the nature and cause of the divergence that becomes manifest during development. Each begins as a growth centre near the periphery of the apical meristem, and the initial positional relationships and histological developments are comparable. The critical difference lies in the inherent growth potentials of the two primordia. The apical initials of a macrophyll primordium, for example in a fern, grow and divide vigorously, especially in the tangential plane of the shoot apex, with the result that more or less extensive *marginal meristems* are formed. As these marginal meristems, like the leaf apical cell, are active loci of metabolism, they act as physiological sinks and draw materials from the axis into the primordium. During their period of activity they make a very important contribution to the formation of the lamina and its vascularization. Somewhat similar developments take place in the formation of the leaf in dicotyledons. In marked contrast, the apical initials of a microphyll primordium, for example in *Lycopodium*, do not give rise to marginal meristems: they persist for some time as a small, coherent group of distal meristematic cells, with a relatively small capacity for drawing metabolic materials from the axis, and they eventually give rise to a small lamina traversed by a single vein. In some instances, as in *Psilotum* or the scale-leaves of *Tmesipteris*, the meristematic properties of the initial cells soon disappear and a non-laminate, non-vascularized microphyll results. In this view, the genetical and other factors which control leaf development will include those which determine the capacity of embryonic cells to retain their meristematic properties, i.e. their capacity for protein and other syntheses.

The fact that the embryos and young sporophytes of *Tmesipteris* and *Psilotum* are leafless and rootless has often been cited as evidence that they are simple and primitive vascular plants. In both genera, however, the young sporophyte is nourished by a saprophytic underground prothallus and leaves only begin to be formed when shoots appear above ground. But in strongly developed rhizome branches essentially the same relationship obtains: branches which remain underground are leafless, those which appear above ground develop as green leaf-bearing shoots. Many examples of greatly diminished leaf development in the underground rhizome are known both in ferns and flowering plants. There are several reasons, therefore, why there should be caution and perhaps a suspension of judgement in assessing the primitive or derivative nature of the Psilotales.

The microphylls in *Psilotum nudum* (*P. triquetrum*) have no central midrib or vein, and there is no vascular connexion between the leaf-base and the shoot stele. In *P. flaccidum*, however, a small vascular trace traverses the cortex from a point just below the leaf-base to the shoot stele (Stiles, 1910). In a well-developed shoot of *Tmesipteris vieillardi* (*T. tannensis*), as described by Sahni (1925), the first formed, small scale-leaves near the base of the aerial shoot have vascular traces which are merely strands of elongated thin-walled cells connecting the shoot stele and the leaf-base. On proceeding upwards in the shoot, there is enlargement of the successive scale-leaves and a gradual transition to laminate leaves. These changes are accompanied by a progressive intensification of the vascular development of the successive leaf-traces traversing the cortex, but it is only in the laminate leaves that the vascular strand extends beyond the leaf-base into the leaf-blade. Even in the fully developed foliage leaf it does not extend to the extreme tip, i.e. into the parenchymatous apiculus. These several facts suggest that the more the meristematic activity of the leaf apex is sustained, thereby constituting a region of active metabolism to which substances are drawn, the greater is the intensity of the vascular development. But where meristematic activity is lost almost immediately after the inception of a primordium, there may be no leaf-trace at all, or only a feeble strand running between the site of the initial meristematic activity, i.e. the leaf growth centre, and the shoot stele. These conclusions are in accord with views already expressed by the writer on the inception of vascular tissue. The conception indicated above may have an interesting application to certain ancient fossils. Among the various microphyllous '*Thursophyton*-leaves', the small spiny microphylls in *Psilophyton princeps* are entirely devoid of vascular traces; in *Asteroxylon* a strand runs from the base of the microphyll to the shoot stele; and in *Arthrostigma* the microphyll has a median strand which runs from a point behind its apex to the shoot stele.

Lastly, a point of considerable interest and importance which emerges from a study of the parasitic genera *Cuscuta* (Convolvulaceae) and *Cassytha* (Lauraceae), both with 'reduced' leaves and other adaptive features, is that the shoot apical meristem shows no unusual features either in its organization or organogenic activities. It is during the further development of the primordia in the subapical regions that the unusual morphological and histological features make their appearance. The same is true of *Psilotum*. Many experimental studies have shown that, in respect of its organization and reactivity, the shoot apical meristem is a region of remarkable stability. This apparently applies also to the effects on it of certain changes in the genetical constitution, the effect of such changes becoming manifest only in the subjacent regions. Contemporary researches indicate the latter to be the regions of most active growth and metabolism in the apex, and it may be that the genetical factors which restrict the further growth of leaf primordia in species with microphylls or 'reduced' leaves do so by precluding or curtailing certain phases of protein synthesis.

The writer is much indebted to Dr. E. G. Cutter for the use of some of her

preparations and to Mr. E. Ashby for assistance in the preparation of slides and photographs.

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EXPLANATION OF PLATE XVI

Illustrating Professor C. W. Wardlaw's paper on 'Experimental and Analytical Studies of Pteridophytes. XXXVII. A Note on the Inception of Microphylls and Macrophylls'.

(All figures are from untouched photographs.)

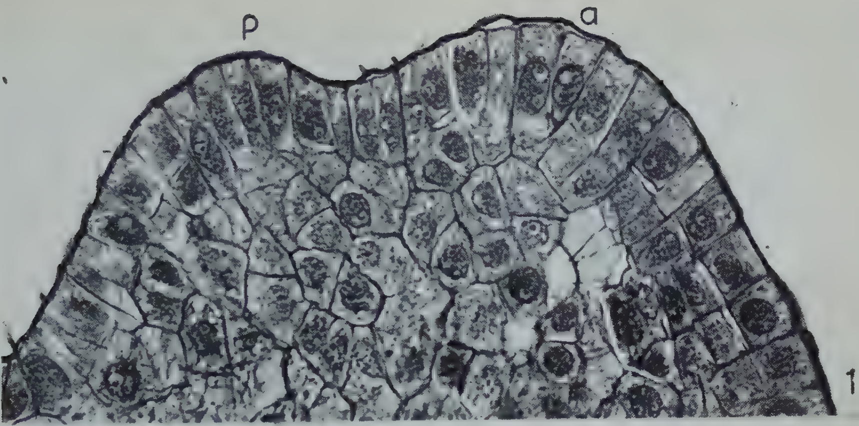
Fig. 1. *Psilotum nudum* (*P. triquetrum*). Longitudinal median section of the last-formed leaf primordium (*p*) and of the shoot apex (*a*). Some of the superficial cells of the primordium have divided by periclinal walls, and there is evidence of considerable cell division in the underlying cells.

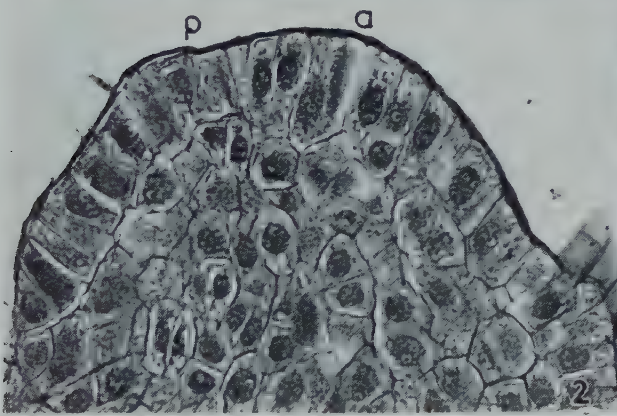
Fig. 2. *Psilotum nudum.* Section as in Fig. 1, showing a very young leaf primordium.

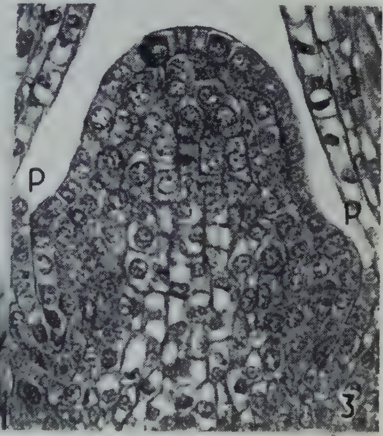
Fig. 3. *Ephedra* sp. Section as in Fig. 1, showing that the young leaf primordium is essentially a multicellular organ, comprising both surface and underlying cells.

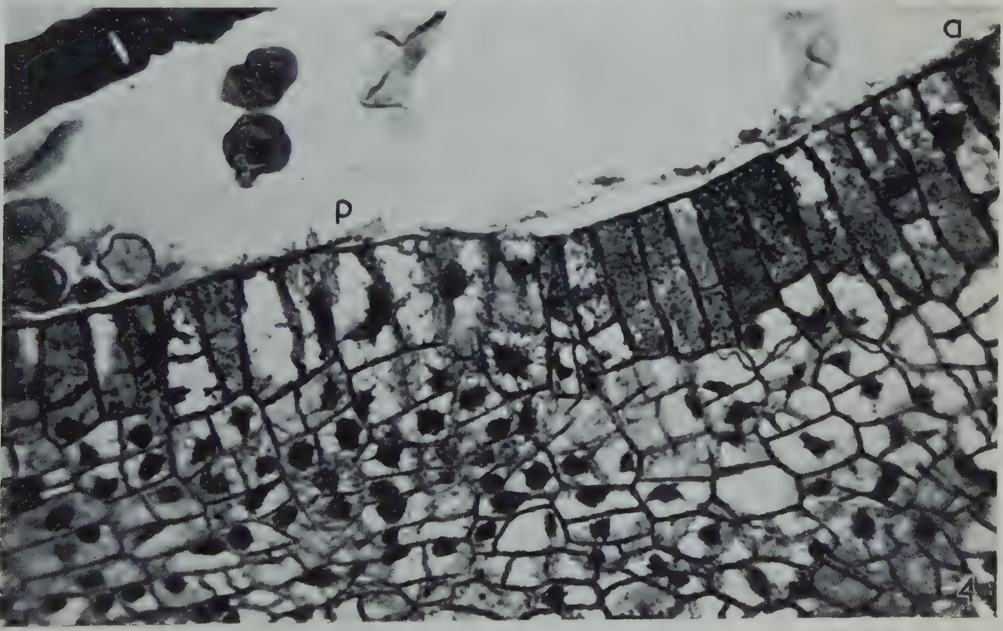
Fig. 4. *Dryopteris aristata.* Section as in Fig. 1, both superficial and underlying cells contributing to the formation of the young leaf primordium.

(All ×225.)









C. W. WARDLAW

Studies on the Physiology of Nodule Formation

VI. The Influence of Bacterial Numbers in the Rhizosphere on Nodule Initiation

BY

HILARY F. PURCHASE
(Pawlett Scholar, University of Sydney)

AND

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With four Figures in the Text

ABSTRACT

The direct examination of the relation between inoculum size and infection rate for the nodulation of a legume is difficult because of the rapid multiplication of the nodule bacteria in the rhizosphere. This has been overcome by (1) using mixtures of normal (virulent) and mutant avirulent strains of clover nodule bacteria, the avirulent acting as a diluent of the virulent, and (2) by employing large original inocula to reduce multiplication. By these means the numbers of virulent clover bacteria were maintained during an experiment at approximately predetermined levels. When a strain of lucerne nodule bacteria was used as diluent the multiplication of clover nodule bacteria was not suppressed.

Nodule number was found to approach the maximum asymptotically with bacterial density; very few bacteria (of the order of 10) were required to give rise to a single nodule only on a plant during an experiment, under conditions where the full nodulating capacity of a root is saturated by a total rhizosphere population of the order of 10^4 bacteria. This relationship supports the view that infection only takes place at discrete foci on the root.

A good fit of the experimental data was given by a compound Mitscherlich curve and by a combination of a normal and a modified Mitscherlich equation. This latter model takes into account the multiplication of sites of infection which occurs in the early seedling stage from foci not occupied at high dilution of virulent bacteria. Multiplication of this kind has also been demonstrated by delayed inoculation experiments.

In the preliminary experiments it was found that comparable rhizosphere stimulation of bacterial growth took place in sand culture, water culture and agar culture and with plants of diverse nodulating habit.

I. INTRODUCTION

MANY studies on nodule formation, including the earlier papers of this series, give little consideration to the possible influence on infection of the numbers of bacteria present in the root surroundings. It is known that even if very few bacteria are added in the original inoculum, they multiply so rapidly in the rhizosphere (West, 1939; Wilson, 1940; Lochhead, 1952) that they soon exceed the number of infections which can be established (Thornton, 1929; McCoy, 1932; Nutman, 1953). Data presented in the

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earlier papers support the view that the limitation to infection is host controlled and suggest that the root is not uniformly susceptible to infection but possesses a limited number of infectable foci. It is, however, still not certain that the origin of the foci of infection may not depend in some way upon the activity or size of the rhizosphere population. Data bearing directly upon this question are difficult to obtain because the number of bacteria which enter the plant for any particular nodule initiation is not known and because of the above-mentioned multiplication in the rhizosphere.

Evidence of serological homogeneity of the bacteria isolated from a single nodule (Hughes and Vincent, 1942) suggests that entry may be affected by a single bacterium or by its progeny colonizing a root hair. Rare cases of nodules containing more than one strain of bacteria (Vincent, private communication) may be due to a number of infected root hairs providing the initial infection as reported by Wipf and Cooper (1940) and Bond (1948), and observed occasionally in the course of the present work.

In general the total number of nodule bacteria in the rhizosphere bears little relation to the number of nodules formed. Perkins (1925) found that maximum nodulation on soya beans took place with as few as 50 bacteria per seed. The relevance of this figure was questioned by Thornton (1929) on the grounds that multiplication would take place and that the initial inoculum does not represent the number of bacteria available for nodulation. Additional direct evidence was obtained by Bhaduri (1951) in water-culture experiments in which the number of nodules on *Phaseolus radiatus* was somewhat increased by a large increase in the size of the initial inoculum. Quispel (1954*a* and 1954*b*) has examined the influence of inoculum size on the formation of root nodules on *Alnus glutinosa*, the causal agent of which does not multiply in the rhizosphere. He found that the number of nodules produced did not increase in simple proportion to inoculum size and concluded that his results supported an hypothesis of infection at discrete foci on the root.

To overcome the problem of controlling the size of the inoculum of nodule bacteria due to multiplication in the rhizosphere, the normal inoculation procedure was modified in two respects: (1) Large original inocula were employed to reduce the amount of multiplication which takes place. (2) The inocula consisted of mixtures of virulent and avirulent strains of nodule bacteria, the avirulent functioning as a diluent of the virulent. By these means it was hoped to maintain the number of virulent bacteria in the rhizosphere at an approximately predetermined level for the period of the experiment and to obtain direct evidence upon the relation between infection and the density of infecting agents in the rhizosphere.

Experiments in which the number of virulent bacteria were thus controlled provided data with which to examine critically the alternative theories that infection takes place anywhere on the root or only at predetermined foci.

Initially the rates of increase of different strains of nodule bacteria were examined in the root medium in the presence and absence of host plants. These experiments, which are reported first, provided information upon rhizosphere multiplication with plants of very different nodulating capacity and grown under different conditions.

II. MATERIALS AND METHODS

In preliminary experiments a comparison was made of sand culture, water culture, standard plant agar (1·5 per cent.), and a soft plant agar (0·5 per cent.) for suitability for rhizosphere studies. Water culture and soft agar were selected for subsequent experiments. The basic nitrogen-free salts mixture (seedling salt solution, Thornton, 1930) which was used at a volume of 8 ml. per test-tube for each medium was sterilized by autoclaving at 20 lb. for 15 minutes. The inocula were prepared in sterile tap water from young cultures of bacteria grown on yeast-water-mannitol-agar slopes (Nutman, 1946). In the case of the soft agar the inoculum was evenly distributed before the agar was allowed to set. The strains employed were from the Rothamsted collection, viz. for red clover: strain A (virulent effective) and Bart A (avirulent); and for lucerne: strain AH_1 (effective). The plant cultures were sown the day following inoculation with seeds of selected genetic lines (detailed for each experiment) which were surface sterilized before planting. The plant cultures were grown in a glasshouse in racks which provided shade for roots. Periodic watering was carried out under bacteriologically controlled conditions to restore the volume to the original 8 ml.

TABLE I

The Relation between the Total (Haemacytometer) and Viable (Plate) Count

Total	Viable	Total	Viable
$2·08 \times 10^8$	$1·60 \times 10^8$	$5·06 \times 10^2$	$7·95 \times 10^2$
$1·03 \times 10^5$	$1·26 \times 10^5$	$1·02 \times 10^2$	$1·11 \times 10^2$
$1·02 \times 10^4$	$1·31 \times 10^4$	$5·06 \times 10$	$7·75 \times 10$
$5·06 \times 10^3$	$6·10 \times 10^3$	$1·02 \times 10$	$1·70 \times 10$
$1·02 \times 10^3$	$8·55 \times 10^2$	5·06	7·30

Total bacterial counts were made with a haemacytometer and viable counts by plating with yeast-water-agar. Plate counts of *Rhizobium* have been stated to be unreliable (Wilson and Kullmann, 1931), but using the above medium the index of dispersion between replicates plates was found to fit a χ^2 distribution. Also the comparison shown in Table I between total and plate counts of inocula was satisfactory at all densities of bacteria.

Inocula of mixtures of virulent and avirulent bacteria were made up from suspensions counted by both methods. Subsequent estimates of the numbers of virulent and avirulent cells in mixture were made by first determining the total number of bacteria by the plate count. The number of virulent bacteria was then found by diluting the mixture and inoculating 5 aliquots from each dilution to plant cultures and later recording the presence or absence of nodules. The most probable number of virulent bacteria in the original suspension was calculated from McCrady's tables (McCrady, 1918). This method was compared with the plate method for pure cultures of clover and lucerne nodule bacteria and was found to be reliable (Table II).

Counts of the rhizosphere population were performed by placing the whole root into a known volume of sterile water from which dilutions and platings were made (first washing estimate). The numbers of bacteria remaining on the root were estimated by (1) determining the number of bacteria removed in 19 further successive washings and (2) by homogenizing the root in a Waring blendor. The results given in Table III of further counts made in this way show that about half the bacteria are retained by the roots after the first washing. The slight difference between the estimates given by the two methods was not statistically significant. In accordance with these results an average correction factor of 1·9 has been applied to the first washing estimates; this is then considered to represent the number of bacteria present upon the root surface and the adhering film of moisture.

TABLE II

Comparison of Plate Count and Plant Test in Estimating Virulent Bacteria in Pure Culture

Culture	Sample	Plate count	Plant test
Strain A	1	$2·0 \times 10^8$	$3·7 \times 10^8$
	2	$2·2 \times 10^8$	$1·7 \times 10^8$
Strain AH_1	1	$2·0 \times 10^7$	$3·5 \times 10^7$
	2	$1·3 \times 10^7$	$2·5 \times 10^7$

In sand culture the rhizosphere population was estimated in terms of the dry weight of root plus sand adhering thereto; elsewhere as the water or soft agar adhering to the roots. The latter was estimated as the weight of water or agar removed by thoroughly blotting the roots and has a mean value of 0·02 ml. The coefficient of variation of duplicate samples was 15 per cent. of the mean. The non-rhizosphere population was sampled from the medium after the removal of the plant.

TABLE III

The Numbers of Nodule Bacteria Removed from the Clover Rhizosphere by a Single Washing and by Subsequent Washing or Homogenization

	Nos. of bacteria per ml. rhizosphere ($\times 10^5$)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
In first washing . . .	5,400	8,850	9,400	—
In 29 further washings .	4,918	6,467	5,797	—
In first washing . . .	2,890	4,820	5,750	4,150
In root homogenate . .	3,110	1,965	6,750	7,300

III. EXPERIMENTAL RESULTS

(1) *The multiplication of single strains of nodule bacteria in the rhizosphere*

Experiment I. The influence of the medium and of planting density

Sand culture, water culture, agar slope culture, and soft agar were compared in this experiment. Since agar slope culture and sand culture were not used in later experiments only the results for the soft agar and the water culture are reported in detail. The experiments were carried out with clover (inoculated strain A) and lucerne (inoculated strain AH$_1$) each sown with 1 plant and 2 plants per culture. Non-rhizosphere and rhizosphere counts were made at 7, 15, 22, 38, 56, and 69 days. Bacterial counts were also made from unplanted batches of media maintained under the same conditions. At the end of the experiment the numbers of nodules were counted. The results are given in Fig. 1.

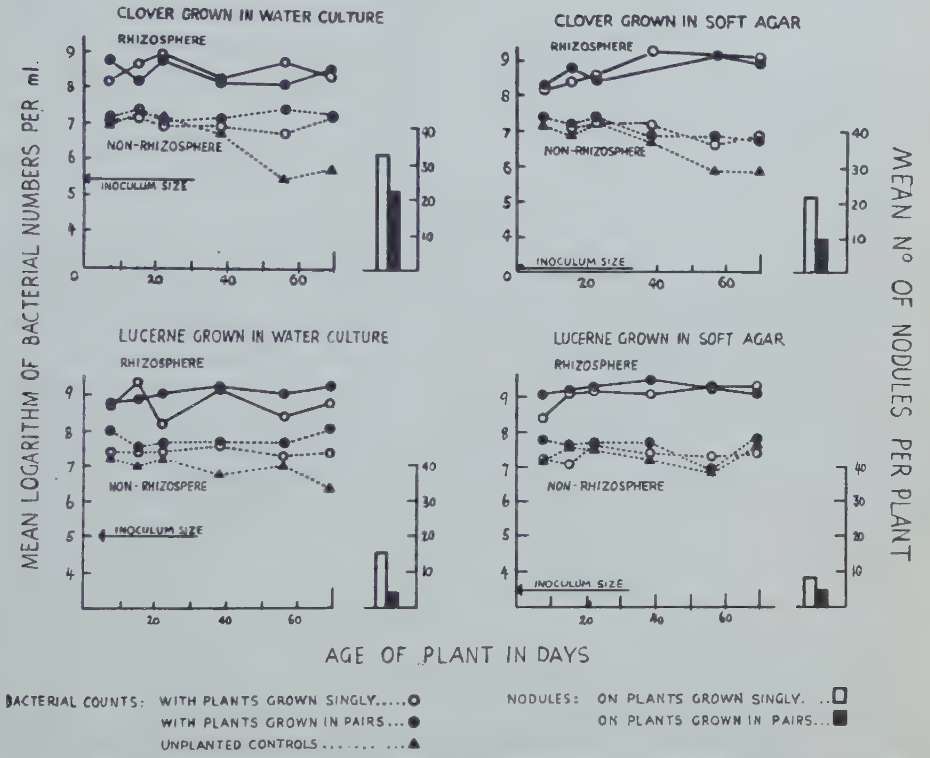

FIG. 1. The relation of the rhizosphere and non-rhizosphere populations of nodule bacteria to the numbers of nodules formed on plants grown in water culture or soft agar.

These results show that with each host, medium, and planting rate, the multiplication of the original inoculum occurred to a much greater extent in the rhizosphere than in the rest of the medium. The rhizosphere and non-rhizosphere populations reached their maxima by the time of the first sample and thereafter remained approximately constant. In accordance with previous experience it was found that numbers of nodules per plant were much less in paired than in single plant cultures. It will also be observed that the non-rhizosphere population in each comparison did not differ significantly from that found in controlled unplanted sets. This indicates that the rhizosphere effect is restricted to the immediate vicinity of the root; an insignificant number of the bacteria arising in the rhizosphere migrating therefrom. The lucerne strain attained a higher level than the clover in both media. This difference occurred even in the absence of plants and therefore is not due to a differential effect of the root secretions on the bacteria.

Very similar results were obtained for sand culture and standard agar with the exception that the size of the populations developing in sand culture was somewhat smaller, and those developing in standard agar a little larger than the populations of bacteria in water culture or soft agar.

These results showed that comparable and stable rhizosphere stimulation occurred with either host under the four conditions of growth employed, and that the fewer nodules found in paired cultures did not reflect a reduction of the rhizosphere effect.

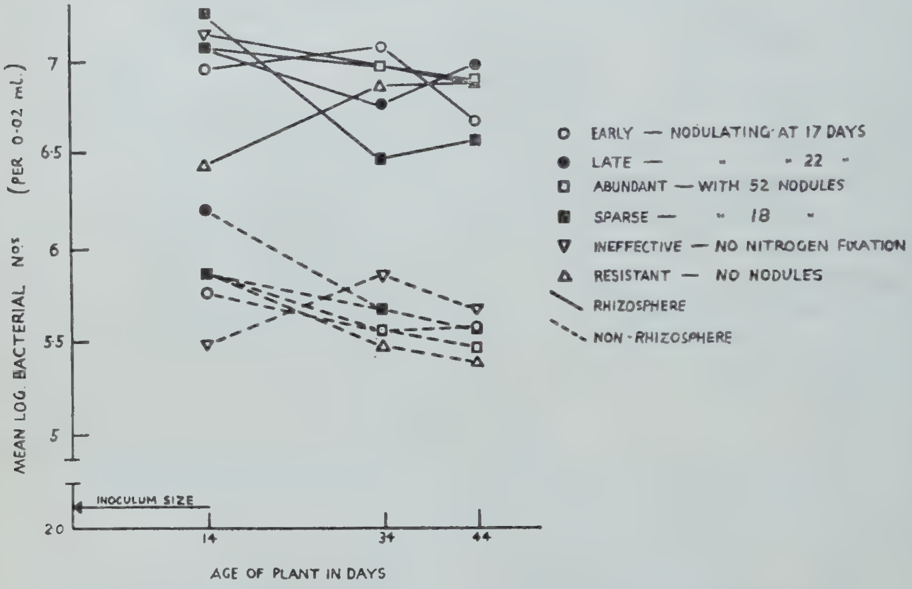

FIG. 2. Rhizosphere stimulation associated with diverse kinds of symbioses.

Experiment 2. The influence of host

The influence of the host was examined by comparing the rhizosphere populations of selected lines of red clover which differed in a number of symbiotic properties. These comprised early and late nodulating selections, resistant, sparsely nodulated and abundantly nodulated lines, and effectively and ineffectively responding lines which were all inoculated with the same strain of bacteria. The number of nodule bacteria in the rhizosphere and in the media are shown in Fig. 2 for each kind of plant together with information on the characteristic properties of the plant material. The experiment was set up in soft agar, 4 replicates per treatment were sampled at each of 3 times.

The results show that the population of bacteria reached a maximum by the time of the first harvest at 14 days and maintained this level until the experiment was terminated at 44 days. The rhizosphere population was everywhere higher than the non-rhizosphere and there were no differences due to plant type. The different lines have therefore the same stimulating effect on the numbers of *Rhizobium trifolii* in pure culture. Moreover, the maximum number of bacteria was attained at least 3 days before nodulation commenced in any set.

This experiment shows conclusively that under these conditions the restriction placed by the host on the number of nodules formed is determined by factors other than the number of infecting agents present.

(2) *Inoculation of mixed cultures of virulent and avirulent nodule bacteria*

The primary object in these experiments was to determine what proportion of the large population of nodule bacteria in the rhizosphere actually takes part in infection of the clover root. To overcome the problem of rhizosphere multiplication a large inoculum of an avirulent strain was employed with which was mixed small numbers of the virulent form as mentioned above. The bacterial strains consisted of strain A as the virulent component and either the lucerne strain AH_1 or the avirulent clover strain Bart A as the avirulent component, Bart A being a stable avirulent variant of strain A. The original total inoculum size in each experiment was of the order of 10^8–10^9 per ml. (or 10^6–10^7 per rhizosphere). The numbers of the virulent component in the inoculum varied from less than 10 to about 10^5 per ml. (or less than 1 to about 1,000 per rhizosphere). Rhizosphere counts were made at intervals and the proportion of virulent bacteria in the mixture determined as described above; nodule counts were also made periodically. The results of the first experiment are given in Fig. 3 and show that the A–Bart A mixture and the A–AH_1 mixture behaved very differently in the rhizosphere and had contrasted effects on nodulation.

The total numbers of bacteria in the A–AH_1 mixtures remained at about the same level throughout the experiment or only increased slightly. In all mixtures, however, the number of strain-A cells increased rapidly and at or before 24 days reached a level equal to that of strain A in pure culture. The lucerne strain was therefore quite unsuccessful in suppressing multiplication of the virulent clover bacteria and unsuited to the object of the experiment. As anticipated from these counts the infection of the root by the clover bacteria was wholly independent of the size of the original inoculum.

These results contrast markedly with those obtained with A–Bart A mixtures in all of which the avirulent component strongly inhibited the multiplication of the virulent, although some multiplication of both strains did occur in all mixtures. In this experiment the average numbers of virulent bacteria per ml. of the rhizosphere varied between treatments initially from less than 1 to about 1,000, and at the end of the experiment from about 500 to 500,000. In contrast to the previous experiment the successful suppression of the virulent bacteria by the presence of a great excess of the avirulent Bart A was associated with the significant depression of nodulation as shown in Fig. 3. This

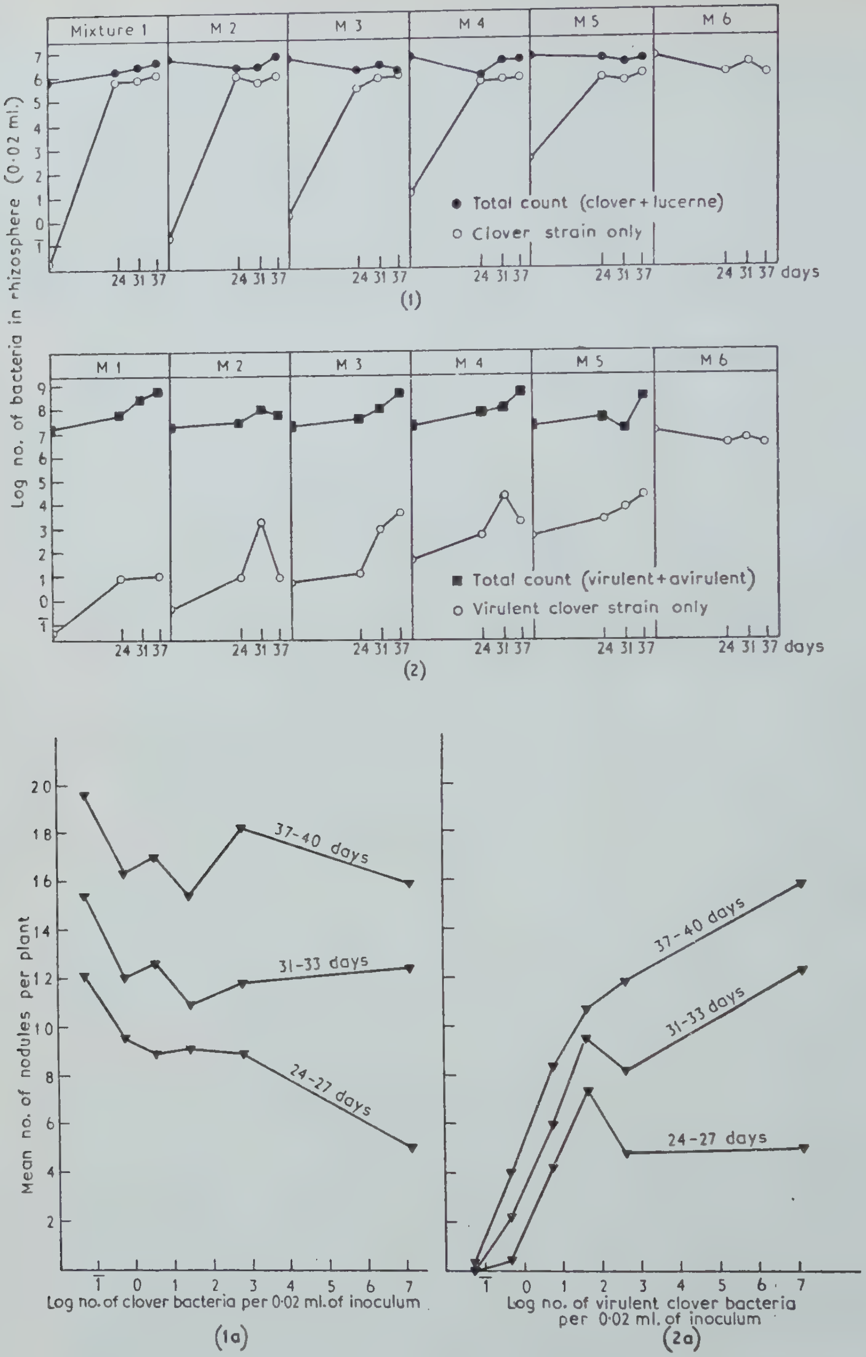

FIG. 3. The relation between the number of virulent nodule bacteria in the rhizosphere and nodule formation on clover (experiment 1). M1–M6, bacterial mixtures.

(1) Rhizosphere counts of mixtures of clover and lucerne strains of nodule bacteria.

(2) Rhizosphere counts of mixtures of normal (virulent) and mutant (avirulent) strains of clover nodule bacteria.

(1A) Nodule formation by mixed inocula of clover and lucerne strains.

(2A) Nodule formation by mixed inocula of virulent and avirulent clover strains.

reduction in the number of nodules produced by high dilutions of bacteria was evident in counts made at about 25, 30, and 40 days.

The reduction of infection obtained in this experiment was not sufficiently well defined to examine in detail the relation between inoculum size and infection, and a further experiment was performed with a larger number of mixtures and increased replication (18 plants per treatment). As in the previous experiment some multiplication of the virulent component occurred as shown in Fig. 4; this was remarkably uniform between mixtures.

The results given in Fig. 4 and Table IV confirm the limitation set to nodulation by the numbers of virulent bacteria in the rhizosphere. It would appear that at most about 400 bacteria per ml. or about 8 in the 0·02 ml. of the rhizosphere would suffice to produce a single nodule on a root during the whole period of test. As the level of the virulent bacteria is increased up to about 500,000 per ml. of the rhizosphere so also does the number of infections which take place, but not in proportion, each additional infection requiring a disproportionate increase in the number of virulent bacteria. Table IV also shows that nodule formation was considerably delayed with the lower concentrations of virulent bacteria.

TABLE IV

The Relation between Bacterial Density and Nodules formed by Mixtures of Virulent and Avirulent Nodule Bacteria

	Log bacterial density per ml.			Time of nodulation. Proportion of nodulated plants		
Mixture No.	Initial x_0	final x_f	Mean number of nodules	at 16 days	at 36 days	Mean number of lateral roots at 36 days
M1	0·86	3·26	4·28±1·06	1/26	18/18	20·1±1·6
M2	1·23	3·99	7·67±1·66	11/25	18/18	17·8±1·6
M3	1·89	4·39	10·28±1·58	16/25	16/18	15·9±1·6
M4	2·04	4·87	15·50±2·65	21/25	18/18	15·7±1·6
M5	2·90	5·33	14·42±1·96	19/24	18/18	12·9±1·6
M6	2·93	5·35	18·83±2·10	25/25	18/18	16·6±1·6
M7	3·79	6·19	25·55±2·97	23/25	18/18	15·1±1·6
M8	4·12	6·47	29·81±3·55	23/24	18/18	14·9±1·7
M9	5·10	6·60	25·22±4·07	26/26	17/18	12·8±1·6
M10	8·20	8·50	22·33±2·90	25/26	16/18	15·4±1·6

DISCUSSION

These results show that the large number of nodule bacteria found in the rhizosphere of the clover plant is greatly in excess of that required for optimum nodulation and again emphasize the dominant role of the host plant in determining infection.

The exact form of the relation between inoculation density and nodulation depends upon the mechanism of infection. The above data give an opportunity, not heretofore practicable with a legume, to examine and to test alternative theories of infection. These may be briefly stated as follows:

(1) The hypothesis of a uniformly susceptible root

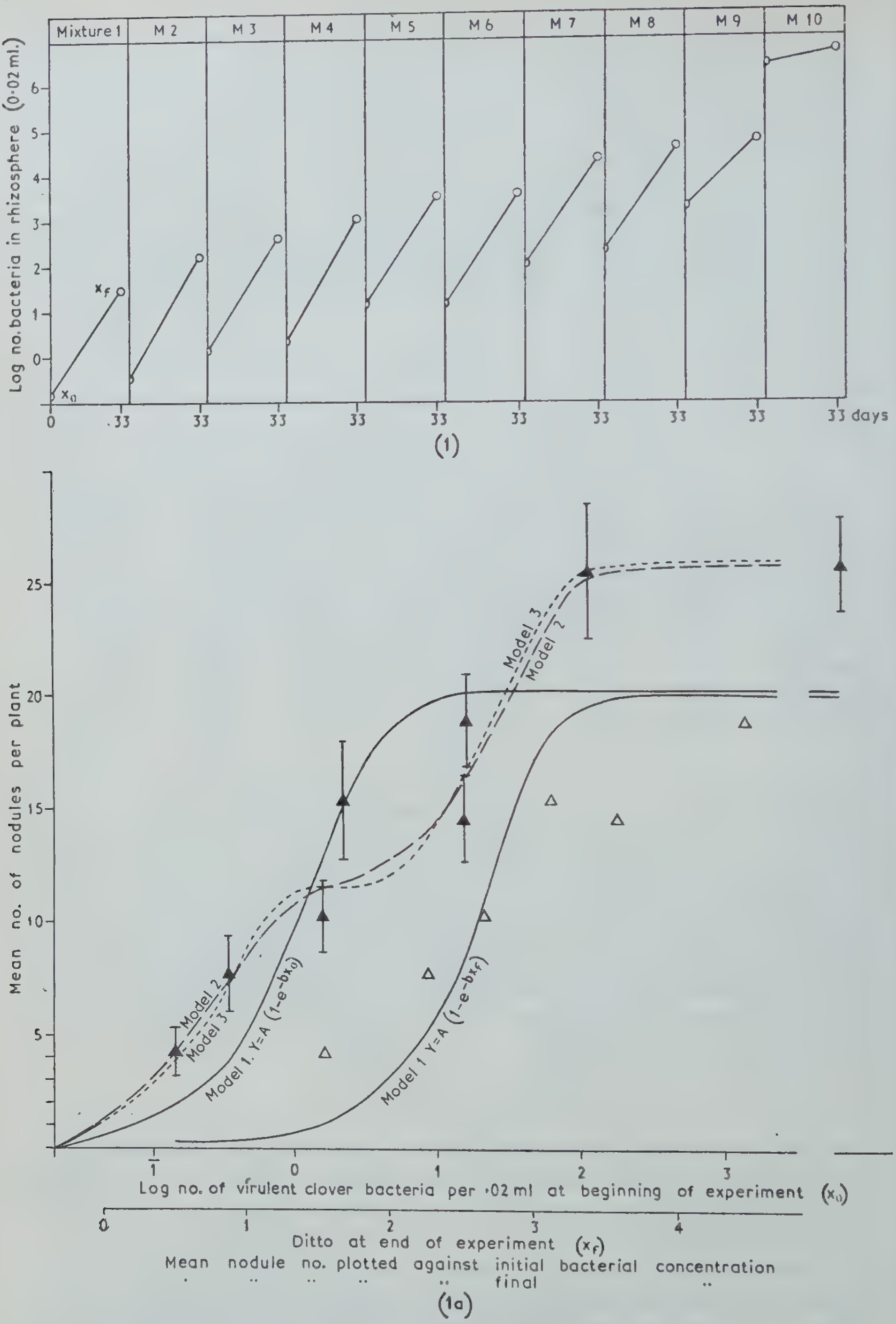

FIG. 4. The relation between the number of virulent nodule bacteria in the rhizosphere and nodule formation on clover (experiment 2). M1–M10, bacterial mixtures.

(1) Rhizosphere counts of the virulent component in mixtures of virulent and avirulent strains of clover nodule bacteria.

(1A) Nodule formation by mixed inocula of virulent and avirulent clover strains. The fit of simple Mitscherlich and compound and modified Mitscherlich curves to experimental data are entered on the figure.

On this view, infection will depend upon the density of the infecting agent and the relation between inoculation density and nodulation will be linear up to a point set by some limiting factor, such as the maximum population which can occupy the rhizosphere. This relation will obtain whether a large or small number of bacteria gain entry at each infection, and whether the inoculum is uniformly virulent or gives rise to only a small proportion of bacteria which are capable of gaining entry and forming nodules. It is clear that the results do not conform to this hypothesis since at no stage is a linear relation disclosed between nodule number and bacterial density.

(2) The hypothesis of infection at discrete foci

On this hypothesis the probability that a site will remain unoccupied at the end of its period of availability is given by e^{-bx} where b is the probability of infection of a site by a specific bacterium over the time that the site is available for occupation and x is the concentration of bacteria. This probability is essentially an integral of the succession of probabilities in time over the period of exposure and would change with the average probability per unit time of exposure and the duration of the exposure. The relation between inoculation density and nodule formation would then conform to the Mitscherlich type of relation.

$$Y = A(1-e^{-bx}) \tag{1}$$

where Y is the number of nodules, x the concentration of bacteria, and A is the number of sites available. This equation will apply if all the foci are exposed either at once or successively provided that b and x remain constant. It is assumed that except for an early period the system is self-regulatory with respect to the creation of new sites. This regulatory mechanism appears to operate by inhibition from the growing points of roots and nodules, the amount of inhibition contributed by an individual nodule depending directly upon its size (Nutman, 1952). There is quite general evidence that size is inversely related to number so that the overall effect on creation of new sites of varying number of established nodules is likely to be small or negligible.

The Mitscherlich relation implies a progressively diminishing number of additional foci infected with increase in bacteria density. This clearly corresponds more closely to the observed results which are plotted in Fig. 4 as Y against log x with Mitscherlich curves calculated by the method of least squares for early (x_0) and late (x_f) estimates of bacterial density. Errors in the estimation of bacterial density were ignored. It is evident from Fig. 4 that the agreement is only approximate; tests of goodness to fit using early or late estimates of x reveal significant deviations from expectation (see Table V). Values of Y exceed expectation at low values of x and are somewhat less than expectation at x_0 values at about 800.

A simple Mitscherlich relation will only hold, however, if (i) the probability of a focus being occupied at a given bacterial density remains constant, (ii) the bacterial density itself remains constant, and (iii) the unoccupied foci have no secondary effect upon the system.

It is clear that bacterial density is changing throughout the course of the experiment, the rate of increase over the effective period of infection being uncertain. However, the trends of the increase in log counts in experiment 1 were linear and since nodulation was not observed until about 10 days after inoculation and then continued at a decreasing rate for the remaining 30 days of the experiment, it is probable that the maximum difference of logarithm of bacterial densities at latest and earliest infection would be of the order of 1·70, the corresponding maximum absolute increase being of the order of 50 to 1. The ratio of mean density in late and early stages is probably not more than 10 to 1.

TABLE V

Fit of Data to Simple and Compound Mitscherlich Relations

(Mixtures M_8, M_9, M_{10} grouped or omitted)

Model 1 $Y = A(1-e^{-bx})$
Model 2 $Y = A_1(1-e^{-br_1x_0})+A_3(1-e^{-dr_3x_0})$
Model 3 $Y = A_1(1-e^{-br_1x_0})+A_1ke^{-br_1x_0}(1-e^{-cr_2x_0})+A_3(1-e^{-dr_3x_0})$

Bacterial nos. per rhizosphere		Nodule nos.				
			Calculated			
Initial density	Final density		Model 1		Model 2	Model 3
(x_0)	(x_f)	Observed	$Y_{(x_0)}$	$Y_{(x_f)}$	$Y_{(x_0)}$	$Y_{(x_0)}$
0·14	36·40	4·28	1·75	1·12	4·01	3·87
0·34	195·44	7·67	3·90	5·32	7·25	7·25
1·56	491·00	10·28	12·62	10·99	11·42	11·56
2·21	1,482·60	15·50	15·15	18·12	11·75	11·49
15·91	4,280·00	14·22	20·10	19·98	16·19	16·33
17·05	4,480·00	18·83	20·10	19·99	16·52	16·69
123·40	31,000·00	25·55	20·10	19·99	25·13	25·41
246·00	59,000·00	25·63	20·10	20·00	25·59	25·65
2,520·00	79,600·00					
602×10^4	632×10^4					
		χ^2	33·14***	32·07***	4·95 (N.S.)	5·36 (N.S.)
		Constants	A 20·1 $b = 0·6350$	A 20·0 $b = 0·00160$	A_1 10·87 A_3 14·72 br_1 3·115 dr_3 0·02815	A_1 9·61 A_3 16·04 k 2·0 $br_1 = cr_2 =$ 1·08635 $dr_3 =$ 0·03415

It is convenient for preliminary exploration to consider whether the data can be accounted for in terms of two Mitscherlich curves using the densities at early infection and at a subsequent stage as independent variates. If the ratio of early and late infection densities to initial density are r_1 and r_3 this relation can be expressed by:

$$Y = A_1\,(1-e^{-br_1x_0})+A_3\,(1-e^{-dr_3x_0})$$

where *d* is the probability of infection at the later stage. The best fit for this type of relation is given in Table V, Model 2 and is plotted in Fig. 4 against initial nodule numbers. The fit is quite satisfactory. The ratio of br_1 to dr_3 is 110. As it can be expected that r_3 is substantially greater than r_1, d must be very much less than b. If alternatively the coefficient dr_3 is ascribed to the early period, the ratio of mean density in early and late stages of say 10 will leave a factor of 10 for late infectivity relative to early infectivity to be accounted for as it is very unlikely that the probability of infection could increase with time. This factor may be within the limits of error if sampling errors in the bacterial count are not neglected.

Turning now to consider the effect of the non-occupation of foci, experiments in which inoculation is delayed and the early formed foci consequently not occupied have shown that such foci may develop into lateral roots which then develop their own series of foci. This substitution of nodules by laterals and multiplication of foci only takes place in the young seedling less than 25 days old. It is presumed that at this stage the overall regulation of foci formation by inhibition is not fully operative so that it is possible for plants with few early infections to secure some advantage from the unoccupied sites.

It has been demonstrated (Table IV) that low inoculation densities in this experiment also resulted in delayed infections and incomplete occupation of initially produced foci. Some of these will then have grown out as lateral roots and cause a permanent increase in the number of infectable foci later arising on the root. Since only a few of the initial foci are known to be concerned in this effect an increase in nodulation due to stimulated root formation would be expected to be most prominent at the highest dilutions of bacteria. Reference to Table IV shows that this expectation was realized, a significantly larger number of lateral roots appearing on the treatments inoculated with high dilutions of virulent bacteria than on the remainder. In view of these results it is considered that any model which excludes the phenomenon of foci multiplication is incomplete.

Let us suppose that the number of foci available in the first stage (viz. without consideration of foci multiplication) is A_1 and the number occupied is

$$A_1(1-e^{-br_1x_0}) \qquad \text{1st stage}$$

The number not occupied is $A_1e^{-br_1x_0}$ and these make possible by multiplication by a factor k of a further contribution of $A_1ke^{-br_1x_0}$ foci for second phase infection.

The proportion of foci at the beginning of the second phase which is not occupied may be represented as $e^{-cr_2x_0}$ where r_2 is the ratio of bacterial density at the second stage to the initial density, so that the number occupied in the second stage is

$$A_1ke^{-br_1x_0}(1-e^{-cr_2x_0}) \qquad \text{2nd stage}$$

The total number of sites occupied at this time is therefore:

$$Y = A_1(1-e^{-br_1x_0})+A_1ke^{-br_1x_0}(1-e^{-cr_2x_0}) \tag{2}$$

Later infections would be accounted for by a further term:

$$A_3(1-e^{-dr_3x_0}).$$

If we assume $k = 2$ (from the consideration that the maximum effect of delayed inoculation is a doubling of the infection rate and for convenience that $cr_2 = br_1$), the best fit of the data to the expression combining all three stages is given in Table V, Model 3. Support for the time sequence of this model is given by the relation of nodule number to bacterial densities at three successive stages in the preliminary experiment (Fig. 3). The earliest observations at 24 days indicated a maximum at an intermediate density which could have been a consequence of multiplication of foci from unoccupied sites. Subsequently, due to a greater increase in nodulation associated with the higher densities, the depression beyond this maximum was progressively eliminated. The ratio of br_1 to dr_3 is now 30 in contrast to 110 for the preceding model. The time sequence now identifies the coefficient labelled dr_3 as belonging to the last stage, with the implication that the probability of infection of a site is much lower in the late than early stage.

The models which have been successfully fitted could apply to two or three stages in time. This does not mean, however, that infection is discontinuous and occurs only at two or three times or periods. A stage rather covers all exposures for which the index to the exponential remains constant.

From this approach to the data it is evident that the relation of nodule number to bacterial density can be accounted for in terms of infection at discrete foci, the foci to be available over limited times, with a changing bacterial density in time and possibly a changing probability of infection at a particular bacterial density with time. An additional modification can be introduced that unoccupied sites, at least in the early stages, could give rise to additional sites and this hypothesis would conform with the data provided the number of sites generated from an unoccupied site is relatively small. This requirement is supported by independent evidence from delayed inoculation experiments. The composite evidence is in favour of the third model which is of course a convenient approximation to a progressively changing system incorporating the same essential features.

SUMMARY

1. The rhizosphere populations of clover or lucerne nodule bacteria are comparable in size in water culture, sand culture, or agar culture and exceed the non-rhizosphere populations by about × 100.

2. The numbers of nodule bacteria (strains of clover or lucerne) developing in the rhizosphere of clover or lucerne from an inoculation made at sowing reach maximum values at about 1 week before nodulation commences.

3. The numbers of clover nodule bacteria developing in the rhizospheres of the following contrasted types of clover were the same: (1) early and late nodulating plants, (2) abundantly and sparsely nodulating plants, (3) effectively and ineffectively responding plants, (4) nodulating and non-nodulating plants.

4. Using a plant test to determine the numbers of clover nodule bacteria in mixtures of clover and lucerne strains it was shown that the clover bacteria

overgrows the lucerne strain even if initially present in small numbers. No differences were observed in numbers of nodules produced on clover by such mixtures.

5. In mixtures of a normal clover strain (virulent) and an avirulent mutant, the avirulent component competed with the virulent parent strain. In mixtures containing a great excess of the avirulent form, the formation of nodules was reduced.

6. Nodule number was found to increase asymptotically to a maximum with increase in bacterial density. A good fit of the experimental data was given by either of two forms of a compound Mitscherlich equation.

7. From the results it was concluded that infection of the root takes place at discrete foci. The experimental data were not inconsistent with the possible multiplication of foci at sites not occupied at an early stage.

ACKNOWLEDGEMENTS

This study was initiated at the Department of Soil Microbiology, Rothamsted Experimental Station, under the helpful supervision of Dr. H. G. Thornton, F.R.S., while the senior author was holding a Pawlett Research Scholarship.

The authors have also much pleasure in acknowledging the helpful criticism and assistance of colleagues in Australia, in particular that of Mr. G. A. McIntyre, of the C.S.I.R.O., Canberra, for developing the mathematical consideration which led to the elucidation of the results, and Mr. M. L. Dudzinski for the statistical treatment of the data.

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The Cytology of some *Athyrium* species from Northern India

BY

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With Plates XVII–XVIII

ABSTRACT

The present investigation deals with the cytology of genus *Athyrium* from Northern India. Eleven species have been studied and comparisons with the previous records are made. All the North Indian species are sexual and there is a lower percentage of polyploidy compared with the species in Ceylon. Their occurrence at high altitudes suggests that the temperate climate may be significant in this connexion. Polyploidy is noticed only up to the tetraploid level and only in two cases.

DURING the past few decades various systems of classification of ferns have been proposed by different authors which illustrate the extent of disagreement that currently exists. The necessity for cytological studies in taxonomy and phylogeny is well known in higher plants, and Manton's (1950) recent excellent publication on the 'Problems of Cytology and Evolution in Pteridophyta' is of particular value for the proper understanding of the basis of speciation and interrelationship in Pteridophyta.

The genus Athyrium is a cosmopolitan one and the cytological investigation of the genus started with three British species by Manton (1950) followed by her work on nine species from Ceylon (Manton, 1953, Manton and Sledge, 1954). In North America three species have been studied by Britton (1953) and Wagner (1955). Only one Indian species, *A. hohenckarianum* Bedd., has been investigated so far (Mahabale *et al.*, 1953). All these species worked out to date have 40 as the haploid number.

MATERIALS AND METHOD

Eleven species have been studied by the writers, of which nine were collected during July–August 1954 from various localities in Darjeeling District (Eastern Himalayas), 27° 3′ N., 88° 18′ E., ranging from an altitude of 1,000–13,000 feet. The remaining two, *Athyrium schimperi* Moug. and *A. tenuifrons* (Wall) Bedd. were collected in 1953 from Mussoorie (Western Himalayas), 30° 27′ N., 78° 6′ E. The former is one of the commonest ferns in and around Mussoorie, forming thick beds in mesophytic places along the road-side extending up to Kaudia. *A. tenuifrons* is rare and occurs only in shady places

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near water in beds of ravines. This species has also been collected on the Darjeeling to Ging road (5,000 ft.). The floor of the Lebong forests (5,000–5,500 ft.), near Darjeeling, is covered with two other species of *Athyrium*, *A. setiferum* C. Chr., and *A. puncticaule* (Bl.) Moore. At higher levels the elegant *A. foliolosum* (Wall ex Clarke) Bedd. needs mention. All along the road-side from Tonglu monastery (8,500 ft.) to Tonglu Dakbungalow (10,000 ft.) and farther to Gairabas (8,000 ft.) beautifully red-striped fronds of this fern hang from the moist rocks. The size of the fronds is often very large, sometimes even more than a metre. *A. acrostichoides* (Sw.) Diels in contrast exhibits a typical basket-like habit, forming thick tufts near Tonglu Dakbungalow. Its reduced pinnae and habit allow it to be easily mistaken from a distance for a *Lastrea*. Still higher, the dominant species under the Rhododendron forest near Sandakphu Dakbungalow (12,000 ft.) are *A. flabellulatum* (Clarke) Tard. and *A. flabellulatum* (Clarke) Tard. forma of which only the former could be found at the right stage for cytological investigation. The rest of the species were also not uncommon. It may be added that *A. oxyphyllum* Hook. occurs as single plants here and there inside the tea plantations at lower levels (1,500–3,000 ft.). For full descriptions &c., reference, should be made to Beddome's (1892) and Clarke's (1880) exhaustive works.

The technique suggested by Manton (1950) has been in general profitably used, though in certain cases fixations in 1:2 acetic-alcohol proved to be more effective; probably it helped in swelling the small-sized-spore mother cells encountered in some species of this region. The small size of these cells has also been noted in the tropics of Ceylon as compared to temperate European flora (Manton and Sledge, 1954). Microphotographs were taken where necessary. Pressed specimens of the species investigated have been deposited in the Panjab University Herbarium, Amritsar.

OBSERVATIONS

The present observations have been summarized in Table I. All the species appear to be sexual since 64 seemingly normal and viable spores were counted within sporangia of the various species. The course of meiosis was critically followed and found to be normal. It is clear that generally the haploid chromosome number is 40, though in two cases it was a multiple, 80. Polyploidy has been encountered only at the tetraploid level. It is interesting that within the limits of what has been called a single species, *A. acrostichoides* (Sw.) Diels, both diploid and tetraploid races have been found. These are being evaluated cyto-taxonomically. *A. anisopterum* Christ has been reported to have $n = c.\ 80$ from Ceylon by Manton and Sledge (1954). The writers have also noted $n = 80$ in plants collected from Tonglu.

Athyrium boryanum (Willd.) Tagawa needs a special mention. Previously the species has been called[1] *Dryopteris boryana* C. Chr. (Ind. Fil., 225, 1905),

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[1] The references for each synonym are taken from Holttum's *Ferns of Malaya*, Singapore, 1954: and some of them have not been seen in the original.

Phegopteris kingii Bedd. (Handb. F.B.I. and Ceylon Suppl. 84, 1892), *Polypodium subtripinnatum* Clarke (Trans. Lin. Soc. Bot., i. 545, 1880), whereas the basic name is *Aspidium boryanum* Willd. (1810). Tagawa (Acta Phtotax, iv. 144, 1935) placed the species under the generic name *Athyrium* and so did Ching (1936, Lignan sci. Jour., xv. 296). Copeland (1947) has newly added this species to the genus *Ctenitis* and he states 'one such aberrant species is *C. boryana* (Willd.) described from Bourbane ranging from Africa to Japan. Christensen has been disposed to associate it with *Athyrium* but its articulate trichomes seems to me to locate it in or with *Ctenitis*.' Holttum (1954) has recently reported this species from Malaya and is of the opinion that 'in anatomy of the species it agrees with *Athyrium* and not *Ctenitis* and in cases such as this where soral shape is not typical, *Athyrium* anatomy is the only sure guide to affinity'. The writers have also noted a close resemblance in anatomy with other species of *Athyrium* investigated here (unpublished). In this connexion it is of great interest to note the remarks of Manton (1950) for the genus *Athyrium*: 'The sum of anatomical characters and chromosome

TABLE I

Species	Locality and altitude	*n*-chromosome number	Fig. no.
Athyrium acrostichoides (Sw.) Diels	Tld; 10,000 ft.	40	1, 3
A. acrostichoides (Sw.) Diels	Tld; 10,000 ft.	80	2, 4
A. anisopterum Christ	Tlm; 9,000–9,500 ft.	80	
A. Atkinsonii var. *Andersonii* Clarke	Tlm. and Tld.	40	
A. boryanum (Willd.) Tagawa, bipinnate*	Spg; 3,000–4,000 ft.	40	5, 7
A. boryanum (Willd.) Tagawa, tripinnate	Th; 8,000 ft.	40	
A. fimbriatum Bedd.	Klp; 9,000 ft.	40	
A. flabellulatum (Clarke) Tard.	Sph–Phl road; 12,000 ft.	40	
A. foliolosum (Wall ex Clarke) Bedd.	Tlm; Tld; Gbs road; 8,000–10,000 ft.	40	
A. oxyphyllum Hook.	Ging, Bdm; 2,000–3,000 ft.	40	
A. schimperi Moug.	Park, Msf, Mussoorie	40	6, 8
A. setiferum C. Chr.	Th; Lbg; Tlm; 6,000–8,000 ft.	40	
A. tenuifrons (Wall) Bedd.	Msf, Mussoorie, and Brh, Darjeeling; 6,000–8,000 ft.	40	

Bdm = Badamtam, Brh = Birch Hill, Gbs = Gairabas, Lbg = Lebong Forests, Klp = Kalpokhri, Msf = Mossy falls, Phl = Phallut, Spg = Sidrapong, Sph = Sandakphu Dakbungalow, Th = Tiger Hill, Tld = Tonglu Dakbungalow, and Tlm = Tonglu Monastery.

The specific locality only indicates where a particular species is easily available. All the localities belong to Darjeeling in the Eastern Himalayas, except where otherwise mentioned.

* Observations by D. S. Loyal.

number are more reliable than are the details of the sorus when taken alone as an index of affinity.' We have assumed, therefore, that the role of cytology is significant, and that this species belongs to *Athyrium*. The basic chromosome number for the genus *Ctenitis* as reported by Manton (1953) and for some species worked out in this laboratory[1] is 41, whereas the genus *Athyrium* has invariably 40 as its haploid number. Both the bi- and tripinnate forms of the species in question show 40 clear bivalents at diakinesis, as determined from a number of preparations, which evidently supports Holttum's belief.

Another point of interest concerns the preponderance of diploids in the Eastern Himalayas and the small percentage of polyploids. This may be taken to agree with Manton's analysis for Ceylon (1953), showing that there are both more polyploids and a greater frequency of higher-grade polyploids in the tropics as compared with temperate Britain and North America. Climatically the Himalayas are both more temperate at the altitudes sampled and more seasonal than Ceylon—as the ferns grow only in the monsoons.

Mahabale *et al.* (1953) pointed out that most of the species of *Athyrium* are apogamous. The cytological aspects of apogamy have only been reported in two tropical species, *Athyrium maximum* and *A. pinnatum*, from Ceylon (cf. Manton, 1953). The former is triploid while the latter a pentaploid. Meiosis is normal and the diploid spores are produced by the failure of premeiotic mitosis. In classic literature (Farmer and Digby, 1907), however, the varieties of *Athyrium filix-foemina*, var. *clarissima* Jones, var. *clarissima* Bolton, and var. *uncoglomeratum* Stansfield are also reported to be apogamous (and afterwards aposporous), although no exact chromosome numbers are known, nor the grade of polyploidy. In the first case the embryo arises as a bud on the gametophyte, while in the other two cases there is a parthenogenetic development of the egg. All these forms are, therefore, apomicts. The rest of the species reported so far are sexual, as are the 11 Indian species recorded by the present writers who are therefore not in agreement with the statement of Mahabale *et al.* (l.c.).

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES XVII–XVIII

Illustrating P. N. Mehra's and S. C. Verma's article on 'The Cytology of some *Athyrium* species from Northern India'.

Fig. 1. First meiotic metaphase in a spore mother cell of *Athyrium acrostichoides* (Sw.) Diels, $n = 40$ (diploid race). × 1,960.

Fig. 2. Similar stage in *A. acrostichoides* (Sw.) Diels (tetraploid race), showing $n = 80$. × 1,460.

Fig. 3. Explanatory diagram. Fig. 1, showing meiosis in *A. acrostichoides* (Sw.) Diels (diploid race), $n = 40$. × 1,960.

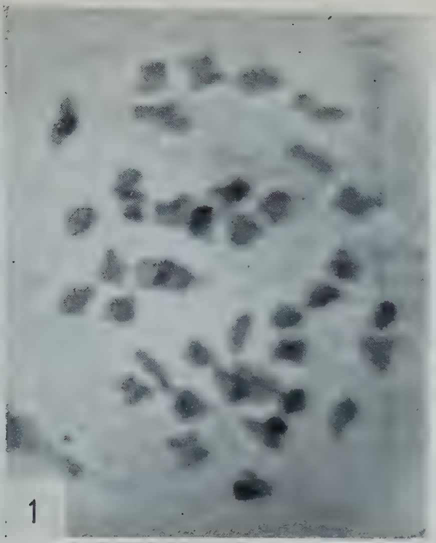
Fig. 4. Explanatory diagram. Fig. 2, showing $n = 80$ in *A. acrostichoides* (Sw.) Diels (tetraploid race). × 1,460.

Fig. 5. Similar stage in *A. boryanum* (Willd.) Tagawa, showing $n = 40$ (bipinnate form). × 1,460.

Fig. 6. Similar stage in *A. schimperi* Moug., $n = 40$. × 1,460.

Fig. 7. Explanatory diagram. Fig. 5, showing 40 clear bivalents in *A. boryanum* (Willd.) Tagawa (bipinnata form). × 1,460.

Fig. 8. Explanatory diagram. Fig. 6, showing 40 bivalents at meiosis in *A. schimperi* Moug. × 1,460.



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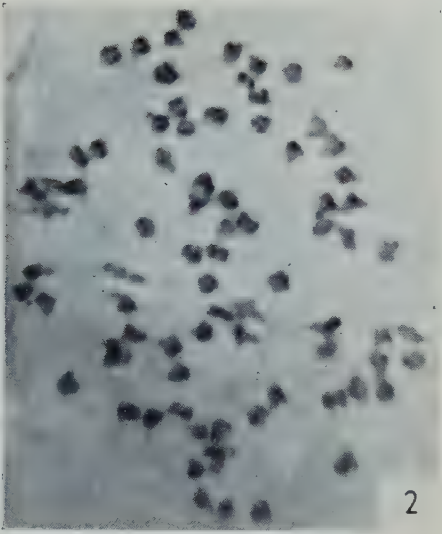

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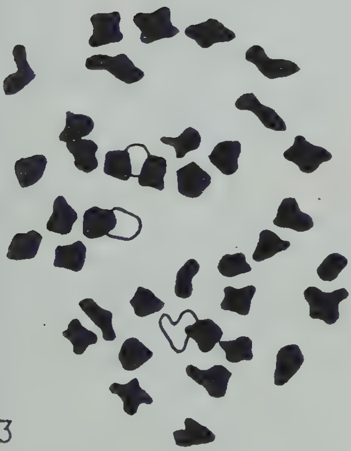

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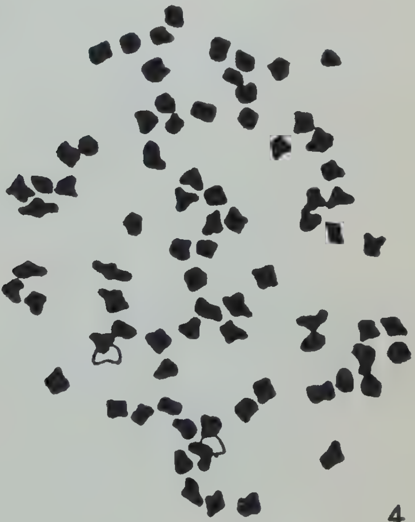

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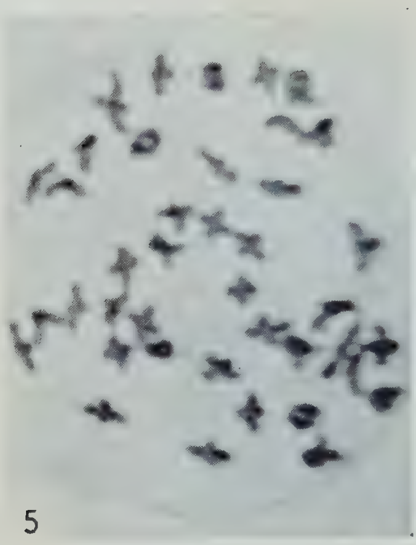
P. N. MEHRA *and* S. C. VERMA



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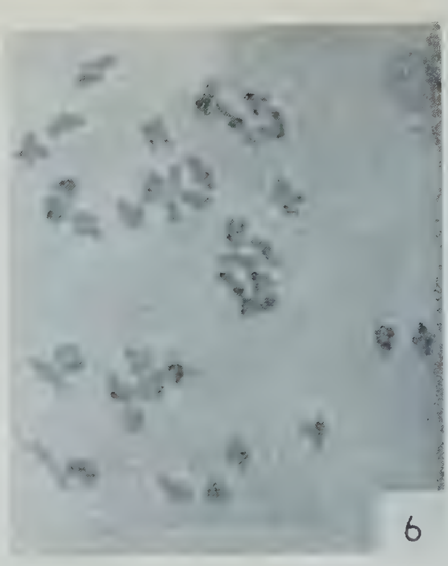

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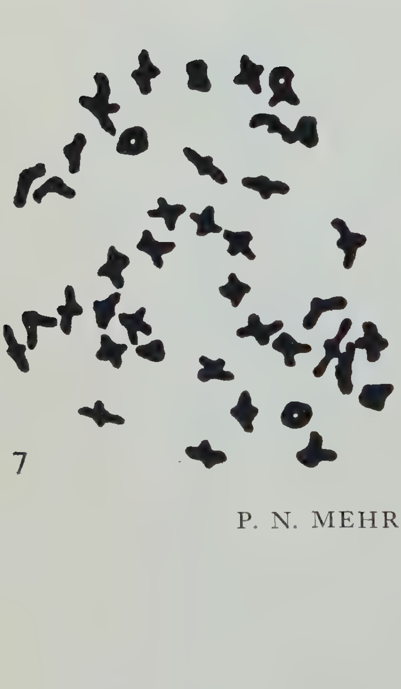

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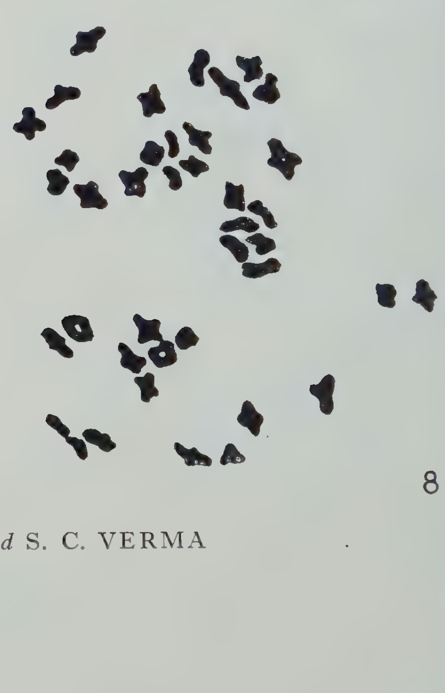

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P. N. MEHRA *and* S. C. VERMA

The Growth of Segments of *Avena* Coleoptiles in Distilled Water and in Indolyl Acetic Acid during Different Months of the Year

BY
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With one Figure in the Text

INVESTIGATIONS were carried out during 1950–1 on the straight growth of coleoptile segments of *Siegeshafer* oats in different solutions of β indolyl acetic acid. It became evident that there were considerable variations between assays in the growth of coleoptile segments both in standard concentrations of IAA and in distilled water. This had been noted previously by Schneider (1938) and Bonner (1933). Whilst this work was in progress Bentley (1950) published a critical study of the possible sources of variation in the responses of coleoptile segments in water and in IAA solutions. Her work did not include a consideration of seasonal variations in responses. The purpose of this communication is to demonstrate that as grain becomes older the straight growth of coleoptile segments from it becomes less reliable as an assay of IAA concentration.

Siegeshafer oats supplied by the Swedish Seed Association were used. The grains were dehusked and germinated in an incubator in red light at 24°–25° C. and humidity 90 per cent. At 67 hours after sowing the coleoptiles had an average length of 1·5 cm. and were ready for sectioning.

Kögl, Haagen-Smit, and van Hulssen using the *Avena* curvature assay showed that there was a diurnal variation in the sensitivity of coleoptiles to auxin (Kögl, 1933; Kögl, Haagen-Smit, and van Hulssen, 1936). In order that the effects of a daily rhythm should be the same in each case, seeds were always sown at 2 p.m. and were ready for sectioning at 9 a.m. on the third day.

All further operations were carried out in a dark room in red light at temperature 20° C. Coleoptiles of length 1·5 cm. were selected and the coleoptiles were severed from the mesocotyls by a sterilized scalpel. Segments of length 9·7 mm. were cut from these coleoptiles at a distance of exactly 3 mm. from the tip. The primary leaf was withdrawn and the segments were mounted on glass threads. Twelve segments on glass threads were placed in 40 c.c. of solution which was aerated by a slow stream of moist sterile air.

Final measurements of the lengths of segments were made in red light with a travelling microscope which measured accurately to 0·1 mm. Fisher's 't' test was used to determine the significance of the differences between means at their final lengths (Tippett, 1941).

Grain of the 1950 harvest was used from January 1951 to October 1951. The

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mean lengths of coleoptile segments after 48 hours in distilled water and in 1 mg./litre IAA are shown in Table I.

TABLE I

Mean Lengths of Coleoptile Segments in mm. after 48 Hours in IAA and in Distilled Water from January to October 1951

Date of assay	Mean length of segments (mm.) after 48 hours in:	
	1 mg./litre. IAA	Distilled water
3·1	19·50±0·88	11·47±0·33
9·1	20·44±1·15	12·08±0·38
17·1	19·75±1·85	12·00±0·41
25·1	19·20±1·34	12·52±0·34
27·2	20·30±1·15	12·40±0·60
4·3	18·71±0·92	11·89±0·42
8·3	19·23±1·13	12·19±0·26
15·3	19·54±1·87	12·94±0·35
5·4	20·18±0·85	12·28±0·62
11·4	20·37±0·78	12·64±0·57
18·4	21·39±1·82	12·97±0·44
26·4	19·71±0·65	12·61±0·53
10·5	19·86±1·30	13·12±0·45
23·5	19·82±1·29	13·33±0·70
30·5	20·49±1·89	13·99±0·48
14·6	20·98±1·20	13·76±0·50
29·6	19·12±1·66	13·83±0·45
5·7	19·75±1·36	13·14±0·55
12·7	20·88±1·31	12·68±0·59
26·7	20·42±1·56	14·18±0·88
6·8	20·23±0·89	13·34±0·18
16·8	19·89±0·77	13·13±0·70
13·9	20·45±1·87	13·86±0·73
20·9	20·47±1·24	14·13±0·44
27·9	19·48±1·15	15·50±0·56
2·10	20·37±1·32	15·00±0·86

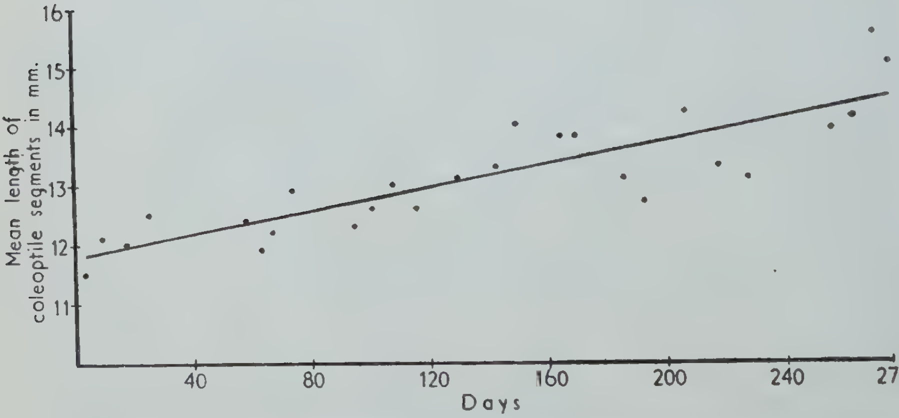

FIG. 1. Mean lengths of Siegeshafer Coleoptile Segments after 48 hours in distilled water from 1st Jan. to 2nd Oct. 1951

In distilled water considerable variation was shown in the responses. In September and October the greatest elongations occurred, final lengths of more than 14·0 mm. being reached. Confirmation of the high final length of coleoptile segments in water during September and October was given by experiments previously performed in 1950 using grain of the 1949 harvest. The responses of coleoptile segments in water from September 1950 to December 1950 are shown in Table II.

TABLE II

Mean Lengths of Coleoptile Segments in mm. after 48 Hours in Water from September to December 1950

Dates of assay	Mean lengths (in order)
15.9 to 22.9	13·05±0·86, 12·27±0·41, 13·72±1·11
26.9 to 16.10	14·14±0·96, 14·33±0·79, 17·20±1·71, 15·19±0·75, 14·13±1·01
23.10 to 29.12	13·76±0·90, 13·21±0·82, 12·54±0·57, 12·38±0·55, 12·86±0·57, 11·74±0·32, 12·53±0·51

The 1951 data (Table I) seemed to indicate a drift towards greater elongation in distilled water as the grain became older. Further analysis of this upward trend was carried out by fitting a linear regression line to the data. This line is shown in Fig. 1 with the experimental data plotted around it. The regression coefficient was highly significant according to the 't' test; the value of t was 8·11 with 24 degrees of freedom. The 5 per cent. fiducial limits of b were 0·007 and 0·012. The linear regression equation was $y = 11{\cdot}77+0{\cdot}0097x$. It was therefore possible to demonstrate a statistically significant upward trend in the elongations of coleoptile segments from January to October 1951.

In the 1951 experiments the responses to IAA were highly variable, the lowest final length being 18·71 mm. in March and the highest 21·39 mm. in April. However, there was no apparent trend towards greater elongation or towards less elongation as the grain became older. The responses in the 1950 experiments were also highly variable and showed no drifts.

It is noteworthy that the upward trend of elongations in distilled water was not continued in November and December 1950 (Table II), and the final lengths of coleoptiles were considerably less than in September and October of that year. Further experimental results are required before this downward trend can be considered statistically significant.

Because there was a drift towards greater elongation of segments in water as the grain became older whilst there was no drift in IAA the differences between the mean lengths in IAA and in water would tend to become less. This means that as grain becomes older coleoptile segments from it gradually become less sensitive to IAA and their straight growth becomes less reliable as an assay of IAA concentration.

Coincident to these results are those of Sweeney and Thimann (1938) who found a seasonal variation in the rate of streaming of protoplasm in epidermal cells of *Avena* coleoptiles. In pure water the rate of streaming increased from November to August and at the same time the stimulations brought about by the addition of auxin became smaller. These present results are also in general agreement with those of Judkins (1946) who found that the age of the *Avena* grain did not affect the magnitude of the curvature produced by standard amounts of growth-promoting substances.

ACKNOWLEDGEMENTS

I am indebted to Professor M. Thomas for advice and criticism throughout the course of the work, and to Dr. J. A. Richardson for assistance with the statistical analyses. The work described formed part of a thesis presented to the University of Durham and was carried out during the tenure of a post-graduate studentship awarded by the Council of King's College, Newcastle.

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An Analysis of Spore Discharge in *Sordaria*

BY

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With Plate XIX and ten Figures in the Text

ABSTRACT

Sordaria fimicola can develop mature perithecia from which spores are discharged when grown in darkness or in light. Under conditions of alternating dark and light (12 hrs.: 12 hrs.) each day, spore discharge is periodic with a low rate during the dark period, succeeded by a gradual rise to a relatively high rate in the light period followed by a decline before the onset of the next dark period. There is no trace of an endogenous rhythm. Transfer from darkness to light always leads to an increase in the rate of discharge, and from light to dark to a decrease. The height of the peak of discharge rate attained in light following a dark period seems to be related to the length of the preceding dark period.

Experiments with light of different colours but of roughly the same energy value show that it is the blue rays that are mainly effective. From cultures of filter-paper yeast-extract medium an orange pigment can be extracted with a maximum absorption, in the visible spectrum, at 470 mμ. It is possible that this is important in connexion with the sensitivity of the fungus to blue light.

INTRODUCTION AND METHODS

IN a number of Pyrenomycetes, so long as the supply of water is adequate, spore discharge from the perithecia tends to be periodic. Some species, such as *Daldinia concentrica* (Ingold, 1946; Ingold and Cox, 1955) are essentially nocturnal, but others, such as *Pleurage* (*Podospora*) *curvula* (Ingold, 1928, 1934) are diurnal. This paper is concerned with an analysis of the phenomenon of periodic spore discharge in *Sordaria fimicola*.

S. fimicola is a common coprophilous species which can easily be isolated and grown in pure culture. In this work a single fresh isolate was used.

The fungus was grown on the following medium:

yeast extract (Difco) . .	4 g.
filter paper . . .	12 g.
agar	24 g.
tap water	1000 ml.

In making this medium the filter paper was dispersed in tap water using an electric blender.

For most experiments the agar was poured into sterilized glass specimen tubes (2·5×2·5 cm.) nearly filling them, and these were contained within sterilized glass capsules.

On the medium used the fungus grows rapidly and perithecia actively discharging spores are produced abundantly in 8–10 days at 18–20° C. Practically no aerial mycelium is formed, a feature of some importance when spore dis-

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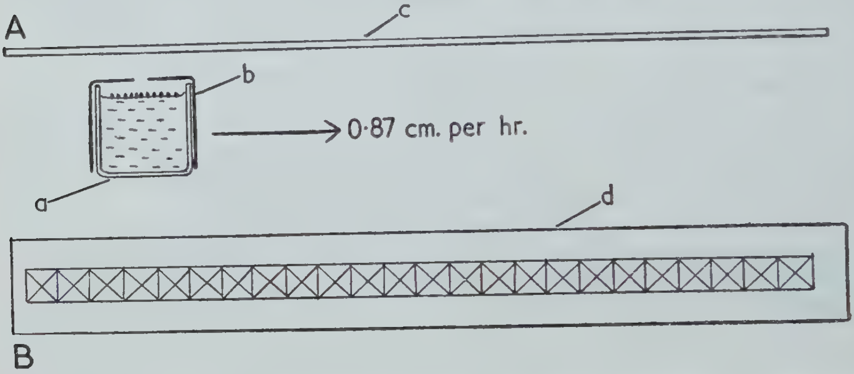
charge is being studied. In some sordariaceous species considerable development of aerial hyphae in culture often interferes with the liberation of spores.

Once discharge starts it continues for about 10 days and at any time during this period all the active perithecia are at almost the same stage of development. There is no progressive ripening of new perithecia. If, however, a culture is examined carefully it may be seen that, in addition to mature perithecia, there are many spherical brownish primordia, but these never develop further.

In *S. fimicola* the spores are discharged to a height of several centimetres. In determining the rate of spore discharge two methods were used. The first will now be explained, but the second will be described later when experiments using light of limited spectral regions are being considered.



TEXT-FIG. 1. A, diagram of 'spore train' apparatus: *a*, specimen tube culture of fungus which moves as shown by the arrow; *b*, aluminium cover furnished with a slot through which the spores are shot; *c*, the graduated slide in sectional view. B, slide in surface view.

This first, or spore-train, method is a slight modification of that previously described (Ingold and Cox, 1955) in connexion with a study of spore liberation in *Daldinia*. A specimen tube with a fruiting culture was covered with a close-fitting aluminium capsule in the top of which a rectangular slot (0·3 cm. wide) was cut. It was then placed on a modified toy railway carriage running on a short length of rail and towed along at a constant and very slow rate (0·87 cm. per hour) by a thread attached to the grooved circumference of a pulley wheel fixed on the axis of a clock. The tube was so placed that the slot was at right angles to the direction of movement of the carriage. Arranged a few millimetres above the tube was a long glass slide on the underside of which a pattern was etched as shown in Text-fig. 1. The length of side of each square of the pattern was made to equal the distance travelled by the culture in one hour. The hourly rate of spore discharge was estimated by counting for each square the number of spores in four low-power microscope fields. These were chosen by a standard procedure, one from each of the triangular areas delimited by the diagonals of the square.

The spore-train was placed in an incubator at 20° C. which could be darkened, or illuminated by 'daylight' fluorescent tubes giving a light intensity of approximately 100 f.c. at the level of the fungus.

A fresh culture was used for each experiment.

PERITHECIUM PRODUCTION AND LIGHT

Some preliminary tests were made of the effect of light and darkness on perithecium production and spore discharge. Petri-dish cultures were grown on yeast-extract-filter-paper agar and these were placed in a glass incubator at 18–20° C. illuminated from above by twin 'daylight' fluorescent tubes producing an intensity of approximately 100 f.c. A parallel set was grown at the same time in the same incubator, but contained in a light-tight box. Each dish was inoculated with a standard disk inoculum (0·7 cm. diam.) from a young culture. Examination after 10 days showed that fruiting and spore discharge had occurred in both light and darkness. As far as the eye could judge the extent of perithecium production, and also the heaviness of the deposit of discharged spores on the inside of the lid of each dish, was as great in cultures grown in darkness as in those grown in light. However, there was a consistent difference in the pattern of perithecium production in the two sets. In the illuminated dishes the region immediately around the inoculum was richly studded with perithecia, whilst in those that had been in darkness perithecia tended to be scarce in the central region. This difference was also reflected in the spore deposits on the lids of the dishes (Pl. XIX).

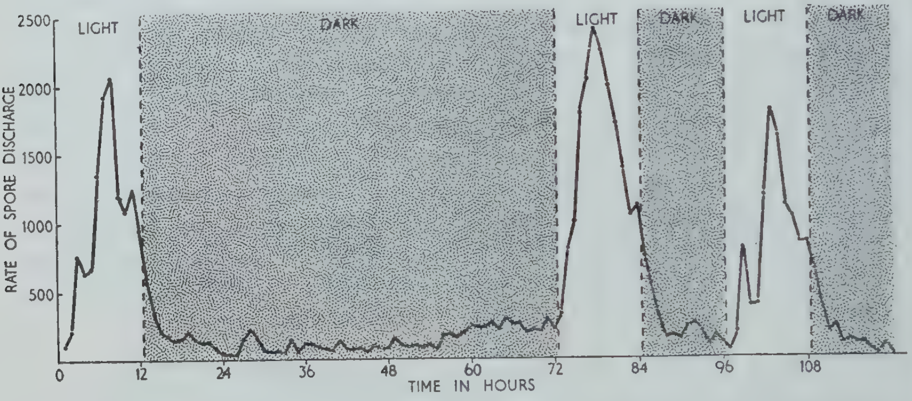
PERIODICITY OF DISCHARGE

The study of spore discharge in *Sordaria* was undertaken largely for the sake of comparison with *Daldinia concentrica*. In that large stromatal pyrenomycete not only is discharge nocturnal under natural conditions, but also this periodicity is maintained for a week or two when the fungus is under conditions of continuous darkness and, for a shorter time, in continuous light. At an early stage in the present investigation it became necessary to know if any periodicity shown by *Sordaria* in its discharge persists after periodic conditions have ceased.

A specimen-tube culture was used which had been grown from the start at 21° C. and subjected to 12-hour light (approximately 100 f.c.) followed by 12 hours of darkness each day. When the culture was 11 days old spore discharge was followed on the spore-train (Text-fig. 2). For the first 24 hours the previous regimen was continued. In the 12-hour light period the rate of discharge rose to a maximum after 7 hours and then declined. In the following 12-hour period in the dark discharge rate declined to a low level. At the end of the first day, instead of the culture receiving light, it was maintained in complete darkness for a further 2 days. Discharge continued at a low rate during this time with no sign of any periodicity. At the end of these 2 days, the culture was again subjected to the conditions it had experienced in the first 12 days of its existence, namely 12-hours' light followed by 12 hours of darkness each day. Spore discharge showed the same type of response as at the start of the experiment: a rapid rise of the rate followed by a decline in the light periods; a very low level in the dark period.

It is thus seen that under conditions of illumination simulating those in Nature, spore discharge is periodic, and *Sordaria fimicola* is a diurnal species, like *Pleurage curvula*, but that there is no suggestion of any endogenous rhythm in discharge which is such a striking feature of *Daldinia*. This general picture has been confirmed repeatedly.

Where an alternation of 12-hours' light followed by 12 hours of darkness is used, the rate of discharge is still falling when light again gives place to darkness. It is of interest to see how far the decline continues if the light period is prolonged.

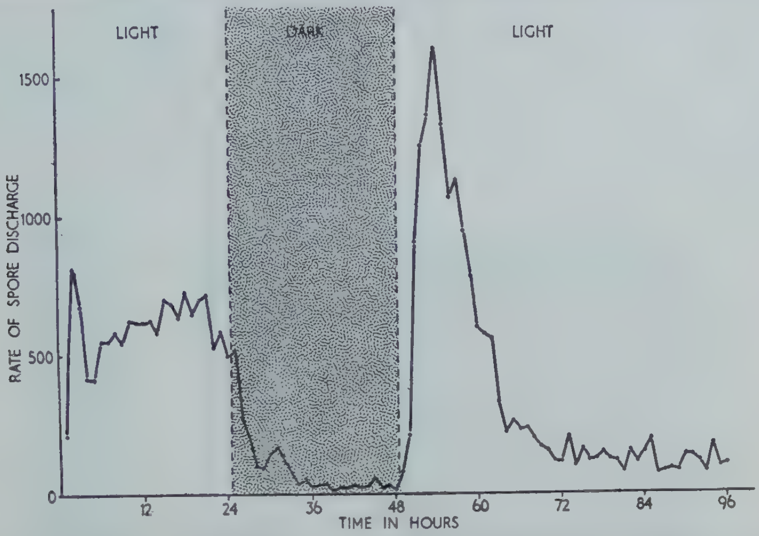


TEXT-FIG. 2. Rate of spore discharge (see p. 466) plotted against time for a culture which has, previous to the start of the experiment, been grown with 12-hours' light and 12-hours' dark in each 24-hour period. Periods of darkness are stippled.

The results of an experiment in this connexion are shown in Text-fig. 3. A culture was used which had been grown for 10 days in continuous light at 21° C. Discharge in the light was followed for 24 hours and during this period the rate was more or less steady at a fairly high level. Transfer to darkness for a day led to a fall to a low level. Return to light resulted in the usual steady increase in spore discharge to a maximum after about 6 hours followed by a fall which continued for some 10 hours. Thereafter for the next 30 hours the rate remained steady at a level considerably higher than that of the preceding dark period, but considerably lower than that originally observed in continuous light.

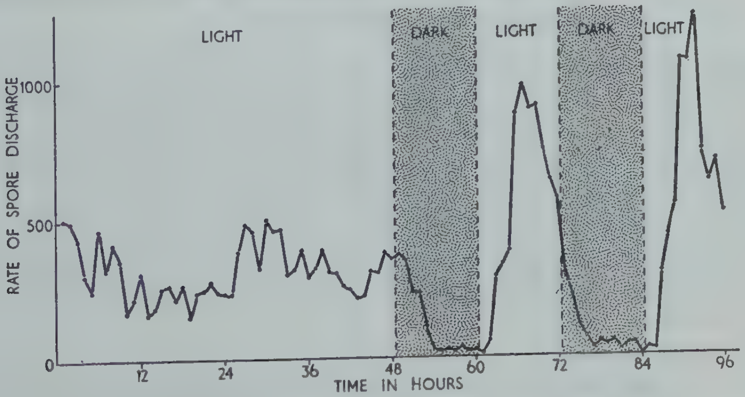
It has been seen that cultures grown in light and in darkness mature perithecia which discharge spores. It is possible, however, that a culture grown in the light responds differently to alternating light and darkness to one raised in continuous darkness. This possibility was studied in two experiments summarized in Text-figs. 4 and 5. In each the conditions under which the cultures were originally grown were maintained for two days before the periodic alternation of 12-hours' light and 12 hours in the dark began.

In both discharge occurred during the continuance of the conditions under which the culture was raised, but without any regular periodicity. However, in both with the onset of the periodic conditions of 12-hours' light and 12 hours' darkness in each day period, spore discharge became periodic.



TEXT-FIG. 3. Rate of spore discharge (see p. 466) plotted against time for a culture grown in continuous light and then treated as shown in the figure.

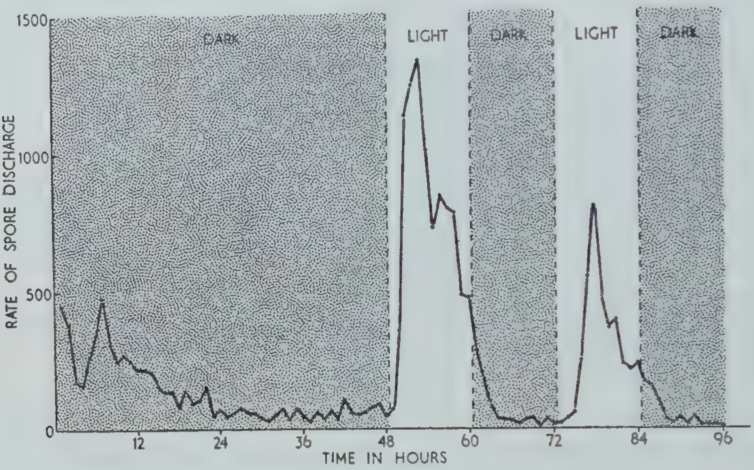
It should be said that the actual levels of discharge are not closely comparable between one experiment and another since the actual number of perithecia in a specimen-tube culture varies considerably.



TEXT-FIG. 4. Rate of spore discharge (see p. 466) plotted against time for a culture grown in continuous light and then treated as shown in the figure.

The work so far described suggests that although light is not a necessity for spore discharge, it has a markedly stimulatory effect since transfer from darkness to light always results in a striking increase in the rate of spore liberation. The experiments also consistently show that in a light period following a dark one a peak of discharge-rate is reached, after a number of hours, followed by a fall. Any explanation of periodic spore discharge must take these striking facts into consideration.

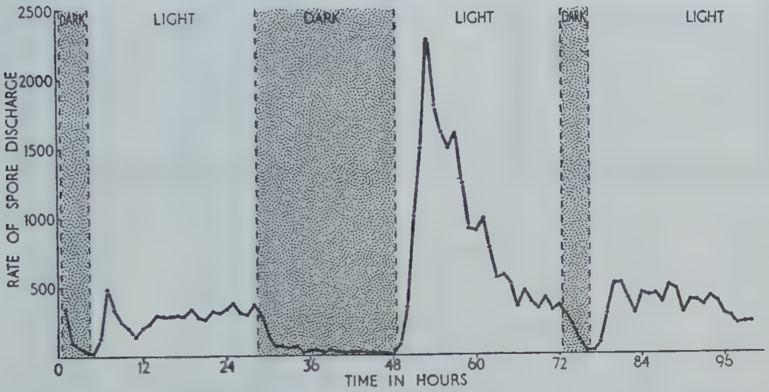
As a working hypothesis it is suggested that spore discharge is stimulated by a substance produced in the light (or that light destroys an inhibitor). It is to be noted that on transfer to light the effect is not immediate but gradual. This is consistent with the idea of a substance being steadily accumulated (or destroyed) as the result of a photo-chemical reaction. To explain the decline following the peak in the light, it is suggested that, at the higher rates, discharge may be limited by the supply of ripe asci. Perhaps in continuous light equilibrium is ultimately reached and discharge is limited by the rate of maturation of asci. In darkness with low rates of discharge it may be that discharge is limited by the speed of the final stages of ascus elongation and rupture and not by the reserve of ripe asci which, therefore, tend to accumulate. Thus when light is again available high rates of discharge can occur without at first being limited by the supply of mature asci. If this theory is correct the height of the peak of the spore discharge curve after a dark period might be expected to bear some relationship to the length of previous dark period.



TEXT-FIG. 5. Rate of spore discharge (see p. 466) plotted against time for a culture grown in continuous darkness and then treated as shown in the figure.

The result of an experiment to test this hypothesis is shown in Text-fig. 6. A 10-day-old culture which had been grown in continuous light at 19° C. was used. It was given a 4-hour period in the dark when the rate of discharge fell to a very low level. This was followed by 24 hours in the light. As usual the rate of discharge rose rapidly to a slight peak followed by a fall and finally a fairly steady rate was established. The light period was succeeded by a dark one of 20 hours when again discharge fell to a very low level. Following this the culture was again illuminated for 24 hours. The discharge rate quickly climbed to a peak which was very much higher than in the previous light period. The rate of discharge then declined. The light period was followed by a 4-hour dark one in which the usual drop to a low level of discharge occurred. Once again the culture received a 24-hour period of light and the course of discharge was very similar to that of the light period following the first short dark period. Further, if we consider the three 24-hour light periods, in the first and third the total spore counts were 6,622 and 8,015, whilst in the second the spore count was 20,506.

It appears thus that not only the height of the peak but also the total discharge in the light period depends on the duration of the previous dark period.



TEXT-FIG. 6. Rate of spore discharge (see p. 466) plotted against time for a culture grown in continuous light before the start of the experiment and then treated as shown in the figure.

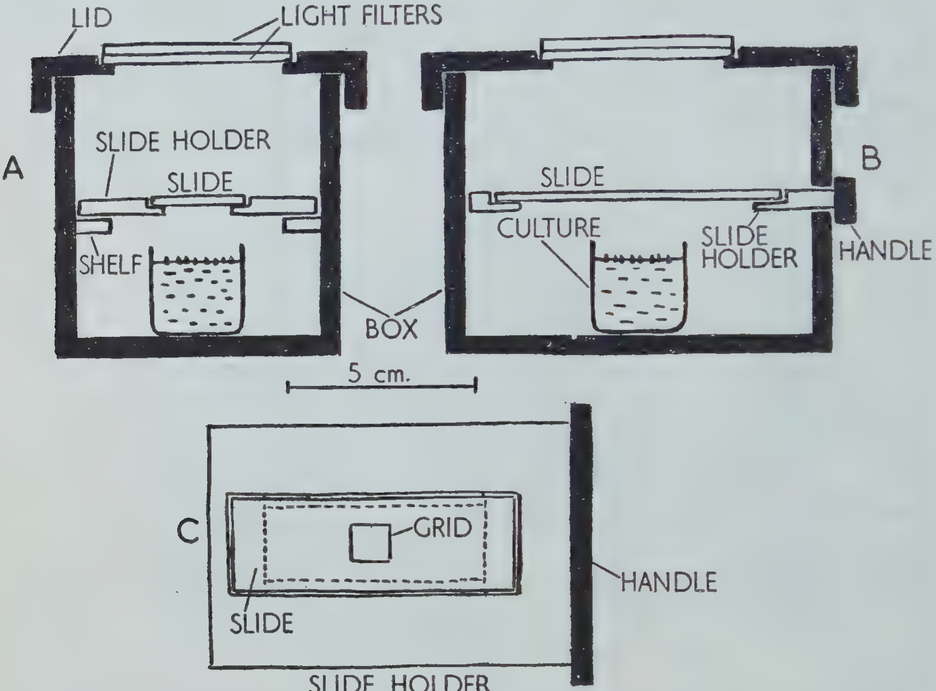
QUALITY OF LIGHT STIMULATING SPORE DISCHARGE

In any attempt to understand a light-sensitive process, it is important to know what spectral regions are most active. In a preliminary study of this problem the response to blue, green, and red light was studied.

The apparatus illustrated in Text-fig. 7 was used. It consisted of a box and lid constructed of black opaque 'perspex'. The fruiting *Sordaria* culture, in a 2·5 × 2·5 cm. specimen tube, was fixed at the bottom of the box. Discharged spores were caught on a glass slide carried by a slide-holder (made of colourless transparent 'perspex') which could be pushed, like a small drawer, through a slot in the side of the box. Within the box the slide-holder rested on two small 'perspex' shelves.

The slide had etched on its under surface a square centimetre subdivided into square millimetres. At the start of an experiment the culture was fixed at the bottom of the box so that the discharging perithecia were directly beneath the etched square. The rate of discharge was determined by exposing a clean slide for 15 minutes and then counting the number of spores deposited on the delimited square centimetre.

Light entered the box only through the square hole in the lid. Here the light filters were fixed. If a coloured filter was used it was always in combination with a square of heat-absorbing glass (Chance ON20) to eliminate infra-red rays. Where complete darkness was required a metal square replaced the light filters.



TEXT-FIG. 7. Box used for the study of the effects of different kinds of light on spore discharge. A, box in vertical transverse section; B, box in vertical longitudinal section; C, slide-holder in surface view.

In these experiments three Ilford colour filters were used. Their characteristics, as given by the makers, are as follows:

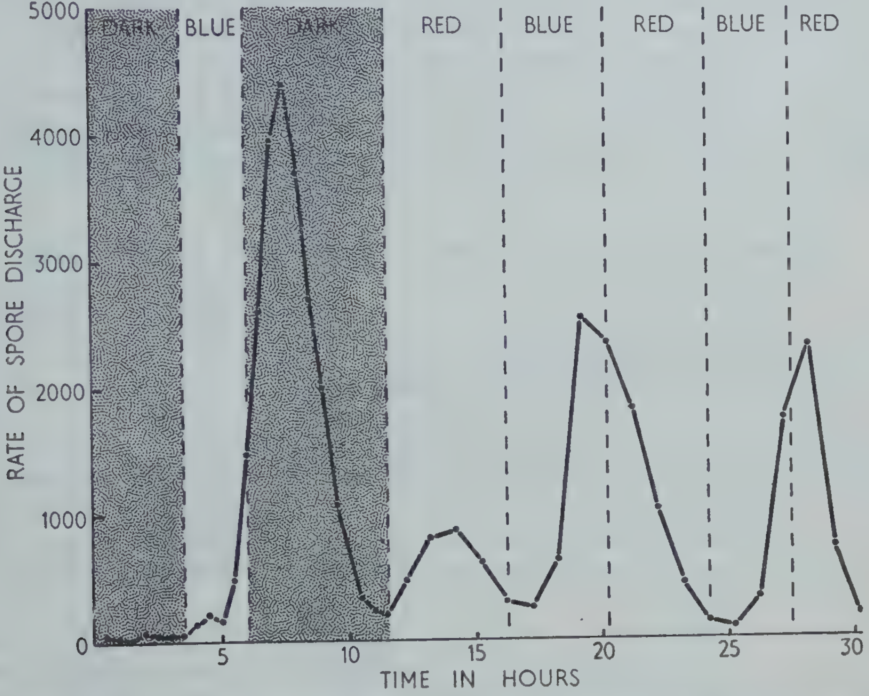
Catalogue number and colour	Transmission range mμ	Maximum transmission mμ
305 (Blue) . . .	350–510	440
404 (Green) . .	480–610	530
205 (Red) . . .	600–720+	650–720+

The light source consisted of two fluorescent 'daylight' tubes. The transmission of energy from this source through the filters was measured using a sensitive thermopile and galvanometer.

Since most light-sensitive physiological processes in plants tend to be influenced by either the red or the blue end of the visible spectrum, the first experiments were concerned with these spectral regions.

The radiant energy transmitted by the red filter plus the infra-red absorbing glass was 2·1 times that passed by the blue in combination with the infra-red absorber.

An 8-day-old culture grown in continuous light at 18° C. was placed in the blacked-out experimental box in the evening. The box itself was kept in the light incubator at 18° C. illuminated from above by the two 'daylight' fluorescent tubes. For an initial period of 3·25 hours the rate of discharge in the dark was followed and found to be low (Text-fig. 8). Then the culture was subjected to blue light. Two and a half hours later the rate of discharge was rising rapidly. Then the box was again blacked-out. The rate continued to increase for an hour and a half, but then fell sharply and several hours later had reached a very low level. Then the culture was subjected to red light. During the next 4½ hours there was a slight rise in discharge rate followed by a fall. Little significance is, however, attached to this temporary rise. At the end of this period in the red, the blue filter replaced the red, and soon a steep rise in discharge rate again occurred. Then the red filter was substituted for the blue and after a few hours discharge had fallen to a low level. Return to blue light again led to a striking increase in discharge rate, but a change to red light brought the rate down to a low one in due course.



TEXT-FIG. 8. Rate of spore discharge (see p. 471) plotted against time for a culture raised in continuous light but kept overnight in the dark before the start of the experiment. The conditions of illumination during the experiment are indicated.

This experiment indicates that subjecting a culture, which has previously been in the dark for some time, to blue light brings about a rapid rise in the rate of discharge after a few hours. This rise can be reversed by darkness.

Further, a change from blue to red light has the same effect as a change from blue to darkness, in spite of the fact that the intensity of the red light is more than double that of the blue.

In later experiments an attempt was made to have light of different colours but of the same energy value. In addition to the blue and red filters of the previous experiment, a green one was also used. With the 'daylight' fluorescent tubes transmission of light through the red and green filters was much greater than through the blue. However, when either the red or the green filter was used in combination with a neutral filter (density 0·50) the energy transmission approximated closely to that through the blue. The comparative energy transmissions of the various system of filters is shown in the following table.

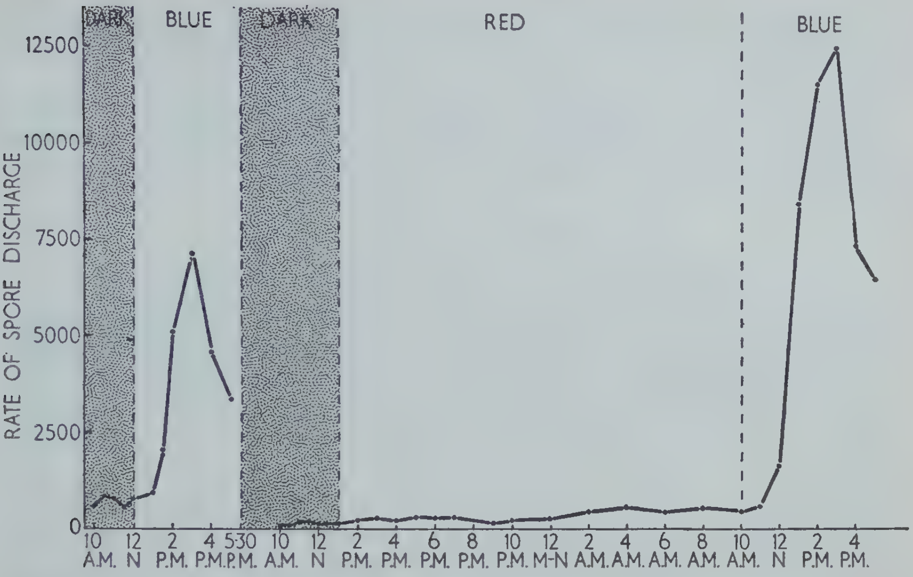
Thermopile Determinations of Energy Transmission in Arbitrary Units

Filter system	Deflexion of galvanometer (mean of four determinations)
Infra-red absorbing filter	8·10
,, ,, +blue filter	0·41
,, ,, +red filter+0·5 neutral filter	0·42
,, ,, +green filter+0·5 neutral filter	0·48

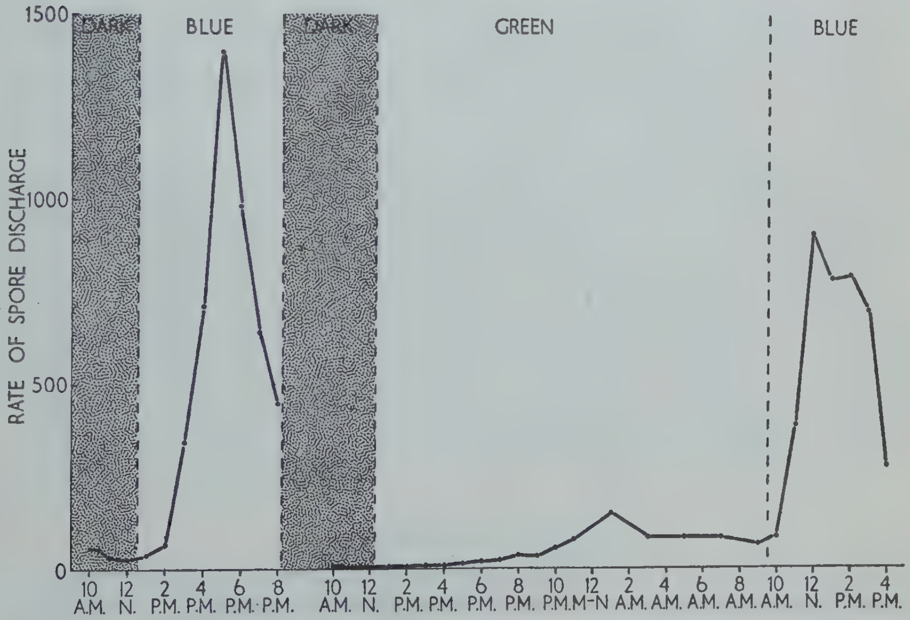
It might be noted in passing that the coloured lights used each represented about 5 per cent. of the total energy of the visible light emitted by the fluorescent tubes. These with the infra-red filter only in position gave a light intensity of 100 f.c. as measured by a photoelectric cell at the level of the fungus.

In the first experiment (Text-fig. 9) a culture which had been grown in darkness at 18° C. for 13 days was used. The spore discharge rate in the dark was low, but on changing to blue light the usual steep rise in rate occurred, after a lag period of an hour or two, a maximum being reached after a few hours followed by a decline. In the evening the culture was again darkened. The object of this first day's test was to demonstrate the ability of the culture to respond to blue light. Next morning the very low rate of discharge in darkness was again followed for several hours, when the culture was illuminated by red light. For 21 hours the rate of discharge in the red was observed. It remained essentially low although there was a slight general rise over the whole period. At the end of this time the culture was again subjected to blue light with the usual result of a very steep rise in the rate of spore discharge. The object of this second treatment with the blue light was to show that the culture had not lost its power to respond.

The second experiment (Text-fig. 10) was exactly similar in design to the first except that green light replaced red for the middle treatment. The course of events was almost the same as in the previous experiment except that in the green light, after a lag period of 12 hours or so, an increase of discharge rate to a comparatively low maximum occurred followed by a fall. Neither in its size nor in the rapidity of its development did this maximum compare with



TEXT-FIG. 9. Rate of spore discharge (see p. 471) plotted against time for a culture raised in darkness and thereafter treated as shown in the figure. From 5.30 p.m. to 10 a.m. during the first 24 hours the culture was in the dark and no observations on rate of discharge were made.



TEXT-FIG. 10. Rate of spore discharge (see p. 471) plotted against time for a culture raised in darkness and thereafter treated as shown in the figure. During the last 14 hours (8 p.m. to 10 a.m.) of the first day the culture was in the dark and no observations on the rate of discharge were made.

that developed in blue light. However, in another somewhat similar experiment the maximum in the green was rather higher although it took almost as long to develop.

There can be little doubt from the experiments recorded above, and from a number of others that are not reported here, that the rate of spore discharge is strongly stimulated by blue light, to a much lesser extent by green, and hardly at all by red.

PIGMENT EXTRACTED FROM *SORDARIA* CULTURES

It is natural to look for some pigment in *Sordaria* which may act as a photoreceptor in connexion with the sensitivity of spore discharge to light. Only very preliminary work on this subject has been undertaken.

It was noted that cultures of *Sordaria* on the filter-paper agar, particularly if they were grown in the light under conditions of good aeration, became yellow-orange in colour. To obtain the pigment the fungus was grown in glass flasks each containing 20 ml. of the usual medium, but with the agar omitted. After 10-days' growth at 18° C. the excess liquid from each flask was drained off, the mat of mycelium with adhering fibres of filter-paper was removed, squeezed fairly dry, and dropped into boiling 95 per cent. ethyl alcohol. By using about a dozen flasks and keeping the volume of alcohol low, a deep orange solution was obtained which after filtration was quite clear.

The absorption spectrum of this clear extract was determined using a Unicam S.P. 500 Spectrophotometer.

So far as the visible spectrum is concerned, there is a single peak of absorption with a maximum at 470 mμ. Between 570 mμ and 900 mμ there is virtually no absorption.

The chemical nature of the orange pigment (or pigments) has not yet been investigated nor has its distribution in the culture. All that can be said is that the high absorption of the pigment in the blue region of the spectrum is consistent with it being the photoreceptor involved in the stimulation of spore discharge. There is, however, one major objection to the theory. Cultures grown in the light produce abundant pigment, but those grown in the dark produce very little. Nevertheless a culture reared in complete darkness responds to light in just the same way as one which, having been reared in light and then given an overnight period in darkness, is subjected to light.

The problem of the photoreceptor is at a very early stage of its exploration.

ACKNOWLEDGEMENTS

Our thanks are due to Dr. W. Ehrenberg for his help in connexion with the thermopile measurements and to Dr. R. A. Shaw for determining the absorption spectrum of the orange solution.

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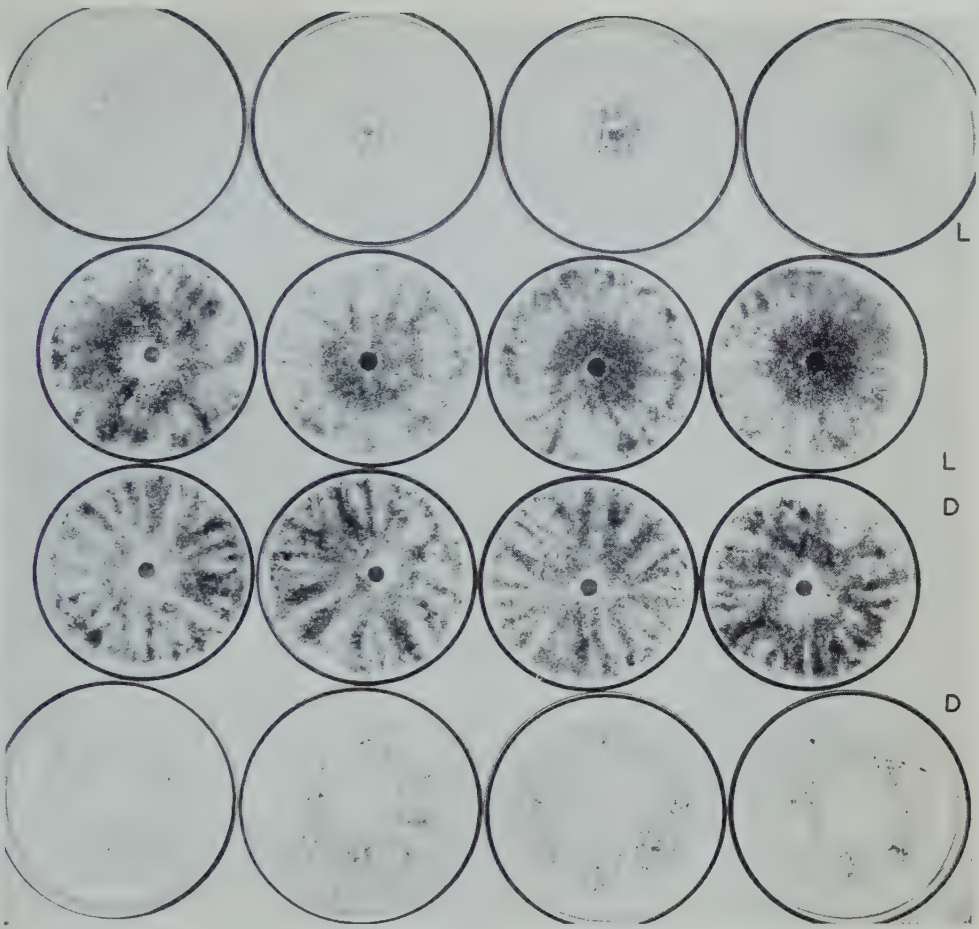
EXPLANATION OF PLATE XIX

Illustrating C. T. INGOLD's *and* V. J. DRING's *article on spore discharge in 'Sordaria'.*

Sordaria fimicola. All cultures 10 days old grown at 21° C. Second row marked L: uncovered petri-dish cultures grown in continuous light (the black dots are the perithecia), the covers being shown in the top row (the black smears are the spore deposits). Third row marked D: uncovered dishes grown in complete darkness; the covers are shown in the bottom rows.



C. T. INGOLD *and* V. J. DRING

An Analysis of Growth and of Form in Young Apple Trees

I. Relative Growth and Net Assimilation Rates in 1- and 2-year-old trees of the Apple Rootstock-variety M.XIII

BY

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ABSTRACT

Two series (A and B) of one-year-old rooted-stems were planted in consecutive seasons and samples were lifted at intervals of a few weeks over periods of one year (A) and two years (B) respectively. The weights at lifting, and their logarithms, were adjusted by covariance with the corresponding values at planting. Net Assimilation Rates, on the leaf-weight basis, showed values and a downward trend similar to those reported for cotton in the Sudan. The values on the bases of the arithmetic and geometric means were rather different and suggested different interpretations. The mean leaf-weight ratios, starting as nil each season, rose to a maximum, never exceeding 20 per cent., by mid-summer. The Relative Growth Rates in the second season (B) started low and fell to a very low value; in the first season of both series they were low from the start—probably because of a check at planting. It is suggested that the low values of the R.G.R. found here, and reported elsewhere for other woody plants, and the low leaf-weight ratios, are due largely to the presence of much residual tissue at the start of the season and that it is the leaf/(total-increment) rather than leaf/(total-weight) that is comparable to the leaf-weight ratio in annuals. It is pointed out, however, that other factors seem to be involved.

INTRODUCTION

YOUNG apple trees are suitable material for the study of total growth—or change in size, and its distribution within the plant—or change in shape. Large numbers are normally raised vegetatively from a single individual, thus eliminating genetic variation, and two of the main tools of the experimental morphologist—grafting and the selective removal of parts by pruning—are normal practices carried out on a large scale. The long life and large final size of the trees make the study of the complete life history difficult and expensive, but the more interesting early, formative years are easy to study.

The long periods of winter dormancy, when little change in size or shape occurs, make detailed linear measurements of the stems possible, and the intervening periods of active growth form natural units, though two trees of the same age are not necessarily in the same physiological state, for one tree may produce its first blossom in the second year of growth, another in the seventh year, and this is evidence of past, and a promise of future, physiological difference.

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The presence of a large residual mass of stem and root at the start of each growing season, and the well-defined distribution of the meristems at that time, are features that can be of importance when studying the factors that influence change in shape.

Changes in form in the apple tree, moreover, follow a general pattern which seems to be governed in part by non-genetic factors such as age, size, rate of growth, cultural conditions, and degree and distribution of pruning; some of these are under control or can be measured. Thus a young tree, starting as a single stem axis, may produce 1st-order branches in its first year, 2nd-order in its second, 3rd-order in its third, but under conditions for more vigorous growth, it may produce 2nd or 3rd, or even 4th-order branches in its first season, and the size and distribution of these laterals may not be the same as in those that arise under conditions of slower growth. Thus two trees of the same size, or two of the same age, may differ in shape owing to differences in past rates of growth or in initial size.

Most of the analyses of growth and its distribution that will be attempted in this paper and its sequels are based on a considerable mass of data collected many years ago from some 1- and 2-year-old trees of a single apple rootstock-variety; this will be supplemented in some instances, however, by data from other material.

Before delving deeper into the complexities of the interaction of the factors influencing the growth-rates of the various parts, it seemed desirable to examine the main data by the well-established methods of growth analysis: this has been attempted here.

MATERIAL AND METHODS

One-year-old rooted-stems from the clonal stoolbeds of the vigorous apple rootstock-variety M.XIII were used. Each stem had grown from a single bud during the previous season: in so far as a bud is analogous to a seed, these trees were analogous to 1-year-old seedlings. There were two series, A and B, planted in consecutive years.

Series A. Consisted of 500 rather small stems with unbranched stems; they were not pruned at planting, but all their roots were removed, so that increments in stem and root could be determined separately. The trees were numbered serially, weighed, and planted in consecutive order at intervals of 3 ft. in rows 4 ft. apart, and with their bases about 6 inches in the soil.

Series B. Consisted of 1,000 rather larger trees, most of which bore branches on the lower part of the trunk. The selection of these trees from ten times their number was not strictly at random, for the largest and smallest were rejected together with those that were damaged or misshapen. The main stem was cut back to about 30 inches from the base—a normal practice—and all branches were removed. The roots were not cut off, but the longer ones were trimmed to 6 inches. An initial sample of 50—5 at random from each 100—was taken to provide a measure of the amounts removed in the various operations before planting and an estimate of the initial relation between fresh- and dry-weights. The remaining 950 trees were numbered serially, weighed, and planted in consecutive order like the previous series.

Methods of Sampling

The intervals between the harvests varied, and the dates were not the same each season, because sampling was not done when the soil was either very wet or very dry—much root is lost under such conditions. The intervals were usually shorter in the growing seasons than in the dormant periods. Each harvest usually took two or three days to complete and the convention has been followed of giving the first date when there were two, and the middle date when there were three. These dates, and the numbers of trees in the sample, are shown in columns 1 and 2 in Table I.

Series A. It was planned to harvest these 500 trees in 10 samples of 50 over 12 months; in practice the last two samples were lifted together and called Harvest 9. For each sample, 5 random trees were selected at the time of planting from each run of 50 (viz. 1–50, 51–100). There were a few dead trees at every harvest and usually a few others with part of the main stem dead. In the preliminary examination (Vyvyan, 1931, 1934), all trees that had made reasonable growth were used, but here, in this fuller analysis, the relative weights and growth-rates of the various organs will be examined and these would be vitiated if trees with much dead wood were included. Every tree with more than 5 cm. of the main stem dead has therefore been rejected.

Series B. It was planned to harvest these 950 trees in 19 samples of 50 over a 2-year period; each sample was to consist of 1 random tree—selected at the time of planting—from each run of 19 (viz. 1–19, 20–38, &c.). In order to double the spacing before the start of the second season, alternate trees—those with 'odd' serial numbers—were chosen for harvests 1 to 9: the remaining 25 trees with 'odd' numbers were included in Harvest 10, the first of the second season. In practice Harvests 11 to 15 consisted of only 25 trees apiece because these were large and complex and took a long time to dismember and weigh. Harvest 19 was omitted. Again there were a few dead trees in most samples, but fewer than in Series A.

Measurements Made

This paper is concerned solely with the total fresh-weights at planting and lifting, and the total dry-weight, and dry-weight of leaves without petioles, at lifting. Many other measurements of weight, length, and diameter were made; it is proposed to describe and discuss these in later papers.

Statistical Methods Used

All the weights at lifting were adjusted by covariance with the initial weights at planting as follows: Let $\bar{x}_1$ and $\bar{x}_2$ be the mean weights at planting for trees used at harvests 1 and 2, and $\bar{x}$ the general mean initial weight of all trees used at all harvests in a series, and let x_1^2, x_2^2, and x^2, respectively be their variances. Let $\bar{y}_1$ and $\bar{y}_2$ be the weights at lifting, y_1^2 and y_2^2 their variances, and x_1y_1 and

x_2y_2 their covariances with the weights of planting. Let n_1 and n_2 be the number of trees used at the two harvests. Let $\bar{y}'_1$, $\bar{y}'_2$, &c., be the adjusted mean values, then:

$$\bar{y}'_1 = \bar{y}_1 + \frac{x_1y_1}{x_1^2}(x - x_1)$$

The square of its standard error, (S.E.)^2, will be:

$$\left\{\frac{(\bar{x}-\bar{x}_1)^2}{x_1^2} + \frac{1}{n_1}\right\}\left\{\frac{1}{n_1-2}\right\}\left\{y_1^2 - \frac{(x_1y_1)^2}{x_1^2}\right\}$$

And the square of the S.E. between two adjusted means, $\bar{y}'_1$ and $\bar{y}'_2$, will be:

$$\left\{\frac{1}{n_1} + \frac{1}{n_2} + \frac{(\bar{x}-\bar{x}_1)^2}{x_1^2} + \frac{(\bar{x}-\bar{x}_2)^2}{x_2^2}\right\}\left\{\frac{1}{n_1+n_2-4}\right\}$$

$$\left\{y_1^2 + y_2^2 - \frac{(x_1y_1)^2}{x_1^2} - \frac{(x_2y_2)^2}{x}\right\}$$

In the same way, the values of the logarithms were adjusted by covariance with the logarithms of the initial weights.

Methods of Growth Analysis Used

If W_0 is the initial weight of the plant and W_1, W_2,...W_t its weights at the end of 1, 2 ... and t weeks, the quotients W_1/W_0, W_2/W_1 and so forth are legitimate measures of the relative rates of growth during the successive weeks. If $W_1/W_0 = Q_1$, $W_2/W_1 = Q_2$, and so forth, then $W_1 = W_0Q_1$, i.e. W_0 has increased Q_1-fold during the week; similarly $W_2 = W_1Q_2$, that is $W_0Q_1Q_2$, and $W_t = W_0\,Q_1\,Q_2...Q_t$. The geometric mean $\sqrt[t]{(Q_1\,Q_2...Q_t)}$ or $\sqrt[t]{(W_t/W_0)}$, which we may denote as Q, will be the mean weekly rate of multiplication in weight for the whole period of t weeks, even if the rate has varied week by week. We may write $W_t = W_0Q^t$, and, of course, $W_0 = W_0Q^0$, but W_1, W_2,...$W_{(t-1)}$ will only equal W_0Q^1, W_0Q^2, &c., if the rate of multiplication in weight is constant week by week.

Relative Growth Rate (R.G.R.)

Although these quotients provide a measure of the relative rates of growth, it is more convenient and usual to use their logarithms. If we denote the logarithms of W and Q by the small letters w and q, we have $w_1 - w_0 = q_1$, $w_2 - w_1 = q_2$, $w_2 - w_0 = q_1 + q_2$, and $(w_t - w_0)/t = q$, where t is the time interval $(t_t - t_0)$ in weeks and q is the mean logarithm or logarithm of the geometric mean quotient Q. If natural logarithms are used, $100q$ is the percentage rate of growth at compound interest, as pointed out by Blackman (1919) who called it the 'Efficiency Index'. This quantity has been called the Relative Growth Rate by West, Briggs, and Kidd (1920), who defined it as follows: '$\log_e W_2 - \log_e W_1 = R/100$, where W_2 is the dry-weight at the end of the week, W_1 the dry-weight at the beginning of the week, and e is the base of natural logarithms.'

Relative Leaf Growth Rate (R.L.G.R.)

The relative growth rate of a part, such as the leaves, is analogous to that of the whole. Thus $\sqrt[t]{(L_t/L_0)}$ will be the mean rate of multiplication in leaf-weight, or area, for the period (t_t-t_0).

The more usual logarithmic form $(\log_e L_t-\log_e L_0)/(t_t-t_0)$ is called the Relative Leaf Growth Rate (West, Briggs, and Kidd, 1920).

Changes in Relative Size and Shape

The relation between the relative growth rates of a part and of the whole and the ratios between the geometric means of their respective weights is simple and obvious. If L_1, L_2, W_1, and W_2 are the leaf-weights and total-weights at times t_1 and t_2, the rates of multiplication will be L_2/L_1 and W_2/W_1 respectively and the ratios L_1/W_1 and L_2/W_2. If $L_2/L_1 = K(W_2/W_1)$, then $K = (L_2/L_1)\times(W_1/W_2)$ or $K = (L_2/W_2)\times(L_1/W_1)$, or in the logarithmic form:

$$k = (\log_e L_2-\log_e L_1)-(\log_e W_2-\log_e W_1), \text{ or}$$

$$(\log_e L_2-\log_e W_2)-(\log_e L_1-\log_e W_1).$$

Thus there is a significant change in the ratio L/W between the G.M.s of the weights if, and only if, there is a significant difference between their relative rates of growth, i.e. if K is significantly different from 1 and k is therefore significantly greater than 0.

The Net Assimilation Rate, or Unit Leaf Rate

The ratio L/W is important because West *et al.* (1920) have shown that the Relative Growth Rate (R) is the product of this ratio and the rate of increase in dry-weight per unit leaf, i.e. $R = (L/W)E$; the term E, which they called the 'Unit Leaf Rate', is the equivalent of the 'Net Assimilation Rate' as defined by Gregory (1917). Their equation for calculating this rate for a period t_1 to t_2

$$E = \frac{W_2-W_1}{L_2-L_1}\times\frac{(\log_e L_2-\log_e L_1)}{(t_2-t_1)}$$

has been widely used, but Williams (1946) has pointed out that the equation

$$\frac{W_2-W_1}{L_2-L_1}\times\frac{(\log_e L_2-\log_e L_1)}{(\log_e W_2-\log_e W_1)}$$

for the mean value of W/L during the period t_1 to t_2 is only valid when L and W are linearly related, and Blackman, Black, and Kemp (1955) have pointed out that the simpler equation

$$\frac{1}{2}\left(\frac{L_1}{W_1}+\frac{L_2}{W_2}\right),$$

which they use, has the same disqualification.

The same would hold true for the further alternative:

$$\frac{\sqrt{(L_1 L_2)}}{\sqrt{(W_1 W_2)}}.$$

These three alternatives all give very similar values.

Most authors have used leaf-areas, but some, from preference or necessity, have used leaf-weights.

Arithmetic and Geometric Means

Most authors have used the arithmetic means and their natural logarithms when calculating relative growth-rates and leaf ratios, but some have used the mean of the logarithms and their antilogs—the geometric means; this method has the advantage that minimal significant differences, or ratios, can be estimated. No authors seem to have given both sets of values, though some, e.g. Williams (1946), have given values from which both can be derived. It is unlikely that the two sets of values will differ widely very often or suggest different interpretations of the results, but this can happen and has done so here. If there is no variance, the A.M. and G.M. are obviously identical. If the values become scattered, however, with a normal distribution about the A.M., this may be altered slightly in either direction, but the geometric mean will always be lower than the A.M. and may be an underestimate. If, however, the values are scattered so that their logarithms are distributed normally about the logarithm of the geometric mean, the G.M. may be altered slightly in either direction but the A.M. will always be higher than the G.M. and may be an overestimate. In practice the distribution in a sample is unlikely ever to be exactly normal or lognormal, at most it will approach one form more than the other. Where there is a large important difference between the A.M. and the G.M., the distribution should be examined to determine which value is likely to be the better estimate. As Medawar (1945) has pointed out, 'there can be no universal solution for the problem of separating the growth from the distribution element in mass curves. Each type requires individual treatment.'

EXPERIMENTAL RESULTS

Correlation Coefficients Between Initial and Final Weights

The correlation coefficients (Fig. 1) between weights at planting and the final weights of the whole tree (including tissues present at planting) are always positive and significant ($P = 0{\cdot}05$), and usually high even towards the end of the second season in Series B where the weight has increased 8-fold. The values for D.W.s tend to be higher than those for F.W.s, the values for increment in F.W. are always positive and tend to become greater towards leaf-fall. The values for the D.W. of leaves are very low, or even negative, at the start of the season, but become positive, and usually high, before leaf-fall. All weights at lifting were adjusted by covariance with those at planting, even when the correlation was low; the ratios between parts were to be examined, all values therefore had to receive the same statistical treatment.

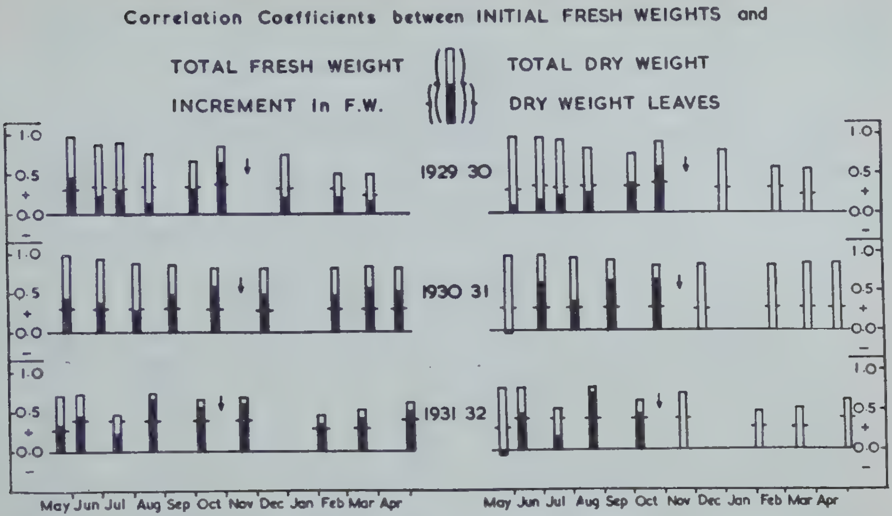

FIG. 1. *Correlation coefficients* between initial F.W.s at planting and (*a*) (on left) total F.W.s and increments in F.W., and (*b*) (on right) total D.W.s and D.W. of leaves at lifting. Arrows indicate leaf-fall, and lateral hooks the minimal values for significance ($P = 0{\cdot}05$).

Total Fresh Weights

Effect of adjustment by covariance. The adjusted total F.W.s (Table I) and their S.E.s are compared with the unadjusted in Fig. 2 A, B, C. Adjustment reduced the S.E.s greatly but altered the mean F.W.s very little—because the initial F.W.s varied little from sample to sample. The relative sizes of the differences between successive values, and the Relative Growth Rates (Fig. 4), were, however, altered considerably.

Series A and B compared. The wide difference (Fig. 2 D) between the final F.W.s for Series A and the corresponding values for Series B were apparently due to the initial differences in weight, for when the values for A are multiplied by the quotient (6·22) between the initial mean values for B and A they almost coincide with those for B.

Total Dry-weights and Dry-weights of Leaves

The arithmetic means of the total dry-weights and of the dry-weights of the leaves (less petioles), both adjusted by covariance with the initial fresh-weights at planting, are given, with their S.E.s, in Tables I and II. The corresponding values of the means of their logarithms, adjusted by covariance with the logarithms of the initial fresh-weights, are given with their S.E.s in the same tables. The antilogs of these mean logs, i.e. the geometric means, are compared with the arithmetic means in Fig. 3 A, B, C, where the values for dry-weight of leaves are plotted on a scale ten times that for the total dry-weights. The A.M.s and G.M.s of the T.D.W.s are very similar in A and almost identical in B and C. The differences between the A.M.s and G.M.s of the D.W.L.s was somewhat greater but was not important except in 1929 (A).

TABLE I

Total Fresh and Dry Weights and Logarithms of Total Dry Weights

Harvest		Number of trees	Total Fresh Weight		Total Dry Weight		Log Total Dry Weight	
Serial No.	Date		Mean	S.E.	Mean	S.E.	Mean	S.E. × 100
Series A								
1	4 Jun.	43	12·6	0·12	6·10	0·08	0·763	0·415
2	1 July	32	15·3	0·39	7·02	0·10	0·827	0·691
3	22 July	34	17·0	0·42	7·87	0·17	0·877	0·902
4	19 Aug.	32	20·0	0·65	9·13	0·54	0·943	1·344
5	3 Oct.	31	23·2	1·29	10·89	0·52	1·009	1·863
6	30 Oct.	27	25·4	1·17	12·31	0·48	1·061	1·662
7	2 Jan.	34	20·8	1·51	10·48	0·73	0·991	2·240
8	24 Feb.	34	21·4	1·22	10·10	0·49	0·973	1·865
9	28 Mar.	69	20·7	0·68	9·67	0·29	0·959	1·172
Series B								
0	10 Apr.	50	65·6	—	34·2	0·26	1·517	—
1	29 May	46	72·5	0·29	33·0	0·28	1·505	0·362
2	1 July	48	89·7	0·97	40·6	0·34	1·596	0·389
3	5 Aug.	49	111·5	1·51	49·2	0·52	1·683	0·472
4	10 Sep.	42	136	2·51	61·2	0·92	1·777	0·643
5	22 Oct.	49	146	3·74	71·4	1·56	1·838	0·921
6	11 Dec.	50	131	2·89	66·3	1·23	1·808	0·338
7	19 Feb.	48	133	2·44	63·9	1·12	1·792	0·732
8	26 Mar.	49	128	2·33	61·4	1·09	1·773	0·879
9	24 Apr.	49	136	2·81	62·8	1·21	1·785	0·831
10	21 May	46	143	3·49	60·3	1·22	1·767	0·906
11	10 June	25	200	6·70	79·7	1·24	1·893	1·248
12	15 July	25	313	12·7	138	5·46	2·127	1·875
13	20 Aug.	25	484	18·3	203	6·81	2·294	1·236
14	7 Oct.	25	499	20·6	248	10·0	2·381	1·889
15	19 Nov.	24	490	22·5	242	11·2	2·364	2·411
16	4 Feb.	48	443	15·6	216	7·63	2·318	1·614
17	16 Mar.	49	486	16·9	238	8·44	2·356	1·676
18	3 May	25	484	24·3	224	11·3	2·337	2·091

* Adjusted by covariance with initial F.W.

The total dry-weights followed the same general pattern as the total fresh-weights but differ in one respect: whereas the F.W.s in Series B increased from 65·6 to 72·5 g. between harvests 0 and 1 in the spring of 1930 and from 135·8 to 142·6 g. between harvests 9 and 10 in the spring of 1931, the D.W.s declined from 34·2 to 33·0 g. and from 62·8 to 60·3 during these periods, and these losses were statistically significant ($P = 0{\cdot}05$). This suggests that the gain by photosynthesis in the young leaves during these periods was insufficient to make up for the heavy losses through respiration during bud-break and early leaf-formation. Similar losses, of course, are normal in germinating seeds; Goodall (1949), who has reviewed past work on this subject, found that Cacao seedlings do not recover the initial dry-weight of their seed for a week after germination or that of their embryos for 4 weeks.

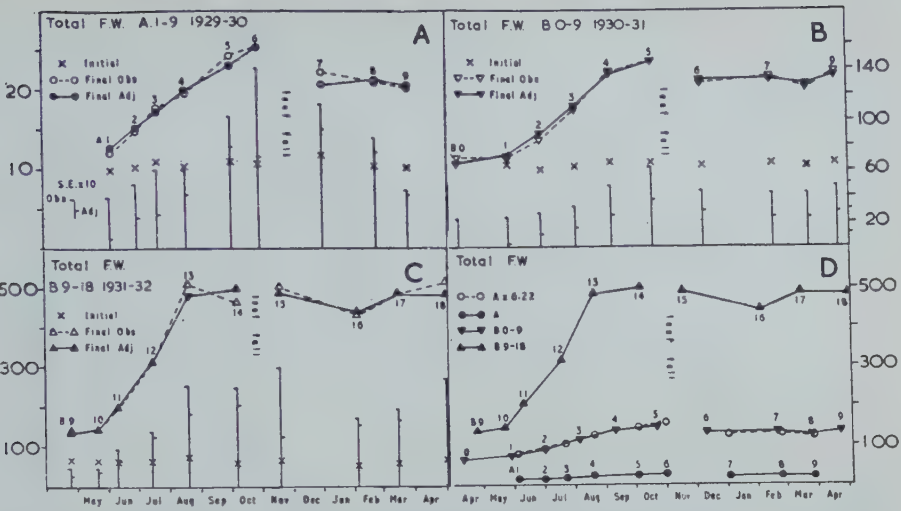

FIG. 2 A, B, C. *Effect of adjustment by covariance with initial F.W.s* on mean total F.W.s at lifting (open symbols denote the observed values, solid the adjusted), and on their S.E.s × 10 (left-hand hooks denote the observed S.E.s, right-hand hooks the adjusted). Mean initial F.W.s shown as crosses (×). D. The adjusted mean values all on the same scale together with those for Series A, multiplied by 6·22—the ratio between the initial F.W.s for Series B and A.

TABLE II

Mean Values of the D.W. Leaves and of the Ratio (D.W.L.)/(T.D.W.) and of their Logarithms*

Harvest No. †	D.W. leaves		Ratio L/T × 100		Log D.W. leaves +2·000		Log (L/T) +2·000	
	Mean	S.E.	Mean	S.E.	Mean	S.E. × 10	Mean	S.E. × 10
Series A								
1	0·21	0·022	3·8	0·39	0·962	1·339	0·199	1·350
2	0·63	0·054	9·2	0·74	1·671	1·076	0·845	1·041
3	0·92	0·066	11·8	0·60	1·914	0·377	1·037	0·297
4	1·17	0·078	12·9	0·54	2·036	0·319	1·092	0·205
5	1·37	0·121	12·3	0·47	2·090	0·365	1·081	0·181
6	1·49	0·108	12·1	0·49	2·135	0·321	1·073	0·178
Series B								
1	1·26	0·069	4·1	0·23	2·029	0·484	0·524	0·503
2	4·12	0·136	10·2	0·30	2·597	0·168	1·001	0·142
3	5·61	0·186	11·6	0·28	2·737	0·149	1·054	0·112
4	7·49	0·208	12·3	0·19	2·863	0·117	1·086	0·069
5	7·57	0·279	10·5	0·20	2·856	0·164	1·018	0·085
10	3·37	0·146	5·8	0·14	2·503	0·251	0·735	0·245
11	12·1	0·518	15·3	0·32	3·072	0·187	1·179	0·090
12	25·9	1·43	18·8	0·34	3·397	0·255	1·270	0·085
13	34·2	1·38	17·0	0·34	3·521	0·164	1·227	0·090
14	29·3	1·65	11·7	0·33	3·444	0·274	1·064	0·134

* After adjustment by covariance with initial F.W. or its logarithm.

† For dates of harvests see Table I.

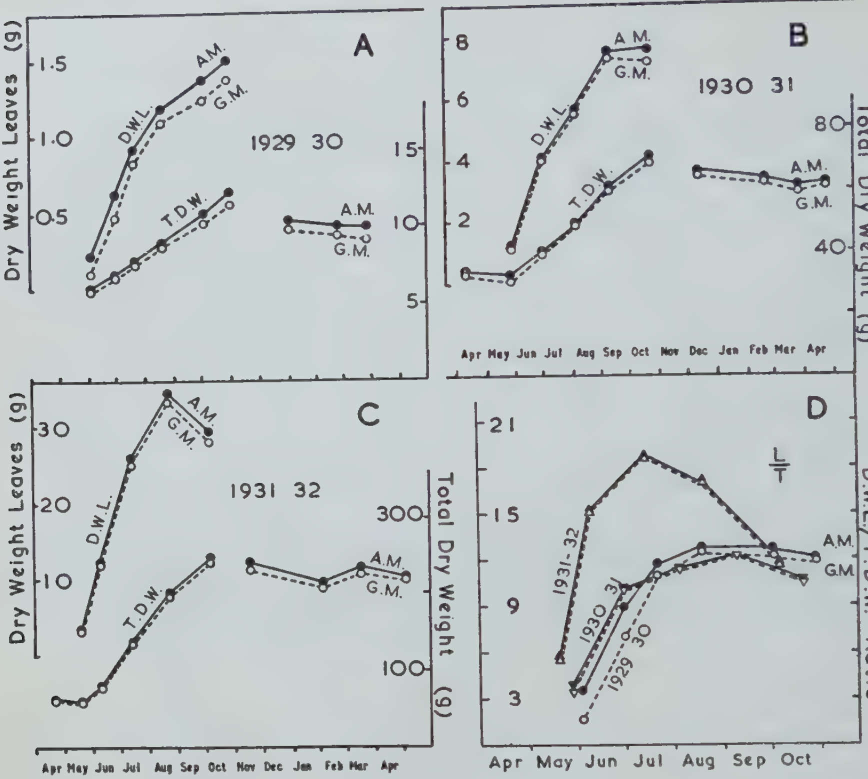

FIG. 3 A, B, C. *Arithmetic* (*A.M.*) *and geometric* (*G.M.*) *means* of the total dry-weights (T.D.W.) and dry-weights of leaves (D.W.L.). D. The A.M.s and G.M.s of the leaf-weight ratio (100 × D.W.L.)/(T.D.W.).

If the dry-weights of leaves, petioles, and developing buds are deducted from the total dry-weights, the mean values for the remainder, of course, showed an even greater decline, suggesting that a considerable part of the dry-weight of stems and roots, at the start of each season, represented reserve food material subsequently used in the building of the young buds and leaves, or in the respiration that provided energy for the building.

The dry-weights of the leaves were, of course, nil at the start of each season when the first leaves were represented as primordia in the dormant buds. Leaf-weight increased from harvest to harvest relatively faster than the total dry-weights; e.g. between harvest 1 and 6 in 1929 the increments in leaf-weight and total-weight were about 7-fold and 2-fold respectively. The rate fell off, or became even negative, towards the end of each season. Inspection of the curves (Fig. 3 A, B, C) suggests that the maximum leaf-weight was attained shortly after the sixth harvest in 1929, between the fourth and the fifth in 1930, and between the thirteenth and fourteenth in 1931. Loss in weight meant that the leaf-fall, mainly from the spurs, was greater than the increase in the number and weight on the shoots.

Leaf-weight Ratio (100 *D.W.L.*)/(*T.D.W.*)

The ratio of the dry-weight of the leaves (without petioles) to the total dry-weight was determined for every tree and the mean values and their S.E.s, after adjustment for the initial fresh-weights, are given in Table II. The adjusted mean values for the differences between the logarithms of the dry-weight of the leaves and the total dry-weight are given also, with their S.E.s. The G.M.s derived from these logarithms are compared with the A.M.s in Fig. 3 D. The A.M.s and G.M.s are almost identical in Series B but differ somewhat in Series A. In general, the leaf-weight ratios were unusually low; the highest value was about 20 per cent., whereas values of 50 to 80 per cent. are normal in most plants. Moreover the values early in the season are usually the highest; here they were the lowest.

The Relative Growth Rates

The relative growth rates, based on the adjusted mean logarithms (Table I), are given in Table III together with their minimum significant differences

TABLE III

Relative Growth Rate, Relative Leaf Growth Rate and their Difference

Interval		Relative growth rate		Relative leaf growth rate		Difference	
Harvests	Days	Mean	Sig. diff.	Mean	Sig. diff.	Mean	Sig. diff.
Series A							
2— 1	27	0·542	0·130	6·046	3·047	5·504	3·041
3— 2	21	0·553	0·251	2·665	2·372	2·112	2·245
4— 3	28	0·546	0·263	0·999	0·801	0·454	0·588
5— 4	45	0·337	0·232	0·278	0·473	−0·059	0·275
6— 5	27	0·445	0·425	0·382	0·814	−0·063	0·436
7— 6	64	−0·253	0·209				
8— 7	53	−0·077	0·245				
9— 8	32	−0·103	0·303				
Series B							
1— 0	49	−0·059	0·047				
2— 1	33	0·634	0·073	3·962	0·706	3·328	0·723
3— 2	35	0·574	0·080	0·921	0·290	0·347	0·232
4— 3	36	0·600	0·099	0·809	0·246	0·209	0·173
5— 4	42	0·337	0·126	−0·038	0·227	−0·375	0·123
6— 5	50	−0·139	0·089				
7— 6	70	−0·051	0·050				
8— 7	35	−0·127	0·150				
9— 8	29	+0·090	0·191				
10— 9	27	−0·146	0·208				
11—10	20	1·446	0·353	6·559	0·845	5·113	0·783
12—11	35	1·540	0·298	2·133	0·419	0·593	0·163
13—12	36	1·065	0·292	0·795	0·394	−0·271	0·159
14—13	48	0·418	0·217	−0·368	0·306	−0·786	0·155
15—14	43	−0·087	0·331				
16—15	77	−0·139	0·170				
17—16	41	+0·216	0·266				
18—17	48	−0·088	0·272				

Sig. Difference ($P = 0·05$)

($P = 0{\cdot}05$). The values for the three seasons are superimposed for comparison on the right in Fig. 4, where they are further compared with the corresponding values (on left) based on the unadjusted mean values of the logarithms. These values, of course, are all on the G.M. basis; corresponding values on the A.M. basis were, however, very similar.

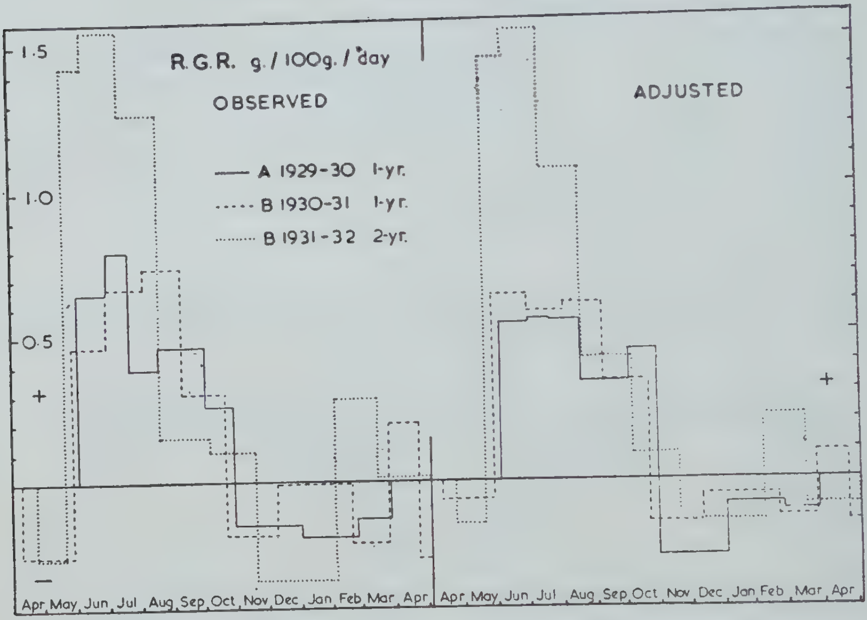

FIG. 4. *Relative Growth Rates* in dry-weight per day, based (left) on mean logarithms of observed values and (right) after adjustment by covariance with logarithms of initial fresh-weights.

There are large fluctuations in the observed values for 1929–30 and 1930–1 and these differ in the two seasons and have no obvious or logical explanation. In the adjusted values (right) these fluctuations have largely disappeared, suggesting that they were due to factors correlated with weight at planting; the values in the two series are now very similar and remain rather uniform throughout the season. The observed and adjusted values for 1931–2 are very alike: at the start they are more than twice as great as those for the previous seasons, but they rapidly decline after July. The low rate of growth in the early part of the first season in each series was probably due to the severe check the trees received when transplanted in the spring. The values are in general very low, but this seems to be characteristic of woody plants (Monselise, 1951). Berry (1938) has reported a very similar rate of multiplication for this rootstock variety.

The Relative Leaf Growth Rates

The G.M. values of the R.L.G.R., based on the adjusted mean logarithms of leaf-weight (Table II), are given in Table III, and those for the three seasons are superimposed on the right in Fig. 5 and compared with the corresponding values on the A.M. basis (left), derived from the logarithms of the adjusted mean dry-weights of leaves (Table II). The values for both seasons (1930–1, 1931–2) of Series B follow the same pattern on both bases, but those at the start of 1929–30 differ widely: on the A.M. basis the values resemble those for 1930–1, but on the G.M. basis they approach nearer to those for 1931–2. Plausible reasons could easily be found for either situation. The values for 1929–30 and 1930–1 both represent those for 1-year-old trees during the first season after transplanting, and 1931–2 those for 2-year-old trees which had been established for a whole season. On the other hand, the Series B trees were severely pruned before their first season (1930–1) of growth but not before their second season (1931–2) and those of Series A (1929–30) were not pruned before planting, and dormant stem pruning will often check leaf growth at the start of the season, though it may increase it or prolong it later in the year (Austin, 1925). As the wide difference between the A.M. and the G.M. was due to high variability, and examination of the scatter showed no special tendency towards either normal or lognormal distribution, it is not possible on the basis of these data to say which interpretation is the more probable.

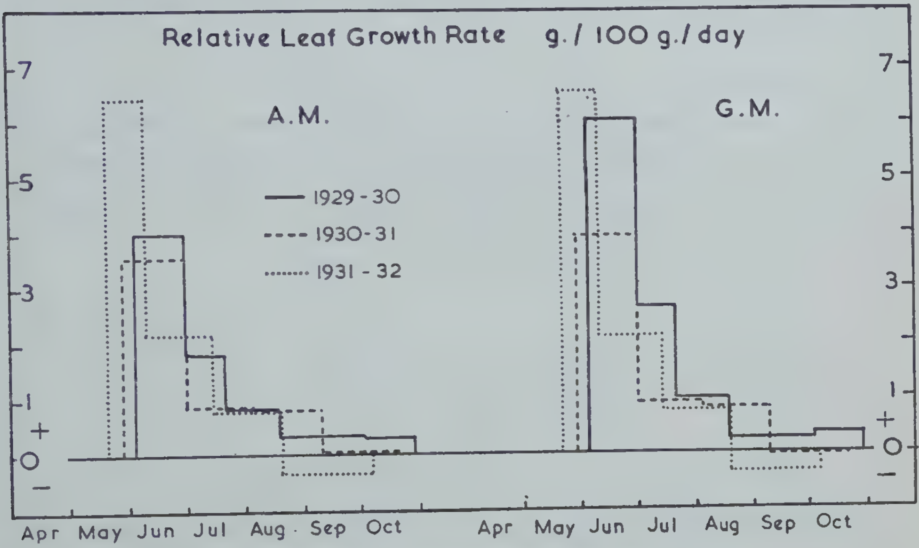

FIG. 5. *Relative Leaf Growth Rates* in dry-weight per day, based (left) on logarithms of the adjusted arithmetic means, and (right) on the adjusted mean values of the logarithms.

Change in Size and Shape

There will be a significant change in the leaf-weight ratio if, and only if, the values for the R.L.G.R. and R.G.R. are significantly different. The differences between these rates and the minimal values for significance

($P = 0{\cdot}05$) are given in the last column of Table III. In Series B the differences, and therefore the changes in the leaf-weight ratio, were significant in every period in both seasons; they were positive at the start of each season and negative at its end. In Series A only the value for the period between harvests 1 and 2 was significant but the lack of significance during the rest of the season was probably due to the high values of the S.E. rather than to low values of the differences.

Net Assimilation Rates

The Net Assimilation Rates on the A.M. and G.M. bases are shown on the left and right respectively of Fig. 6. Both sets of values were calculated by the usual equation $(W_2-W_1)(\log_e L_2-\log_e L_1)/(L_2-L_1)(t_2-t_1)$ but on the left the values W_2, &c., represent the arithmetic means, and on the right the geometric means. These values are on the leaf-weight basis, because it was impracticable to determine the leaf-areas with the man-power available. The values are rather low, and would probably have been lower if Williams's (1946) method of estimating mean leaf-area had been used, but they are not exceptionally low; Crowther (1944) found very similar values for cotton in the Sudan and Egypt. According to his graph (Fig. 5*b*) they fell from about 1·4 to about 0·14 g./g./week during the season, that is 20 g. to 2 g./100 g./day. The values for Egypt were rather lower.

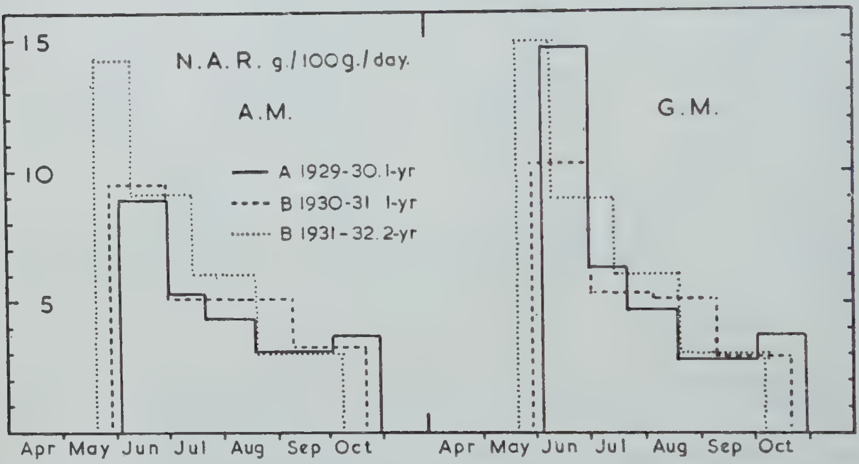

FIG. 6. *Net Assimilation Rates* (grammes per 100 grammes per day). Based (left) on A.M.s of L/T ratios and logarithms of A.M. of dry-weights of leaves, and (right) on G.M.s of L/T ratios and mean logarithms of dry-weights; all adjusted by covariance.

Seasonal Trend in R.G.R. and N.A.R.

There is no consensus of opinion on the nature of the seasonal trend in the N.A.R.; the conflicting evidence and the various interpretations have recently been reviewed by Watson (1952). Where there is a downward trend it may be due to a deterioration in some environmental condition, or to the ageing of some tissues, or to both. When the N.A.R. is based on leaf-weight, as here, it is more influenced by temperature than when based on leaf-area (Blackman *et al.*, 1955), but average temperatures over periods of several weeks are unlikely to have much meaning and therefore have not been worked out here and compared with the values of the N.A.R. It is unlikely, however, that there was a progressive fall in temperature from June to September that would account for the fall in the N.A.R. The average numbers of hours of daylight, from sunrise to sunset, are more likely to have a meaning as they will not be vitiated by fluctuations within the periods, as happens with average temperatures. The mean numbers of hours for the periods between successive harvests were therefore worked out and the values of the R.G.R. and the N.A.R. corrected accordingly; these values are shown in Fig. 7. The downward trend in the N.A.R. has been reduced but not completely eliminated. The residual downward trend may be due in part to an increase in the weight/area ratio and in part to an ageing of the leaves, but this is a factor hard to estimate, for old leaves are progressively lost from the spurs and the bases of the shoots, and new leaves are formed at the apices of the shoots until extension growth ceases. The low value for the N.A.R. during the last period but one in 1929 was probably due to a very dry spell that occurred that summer.

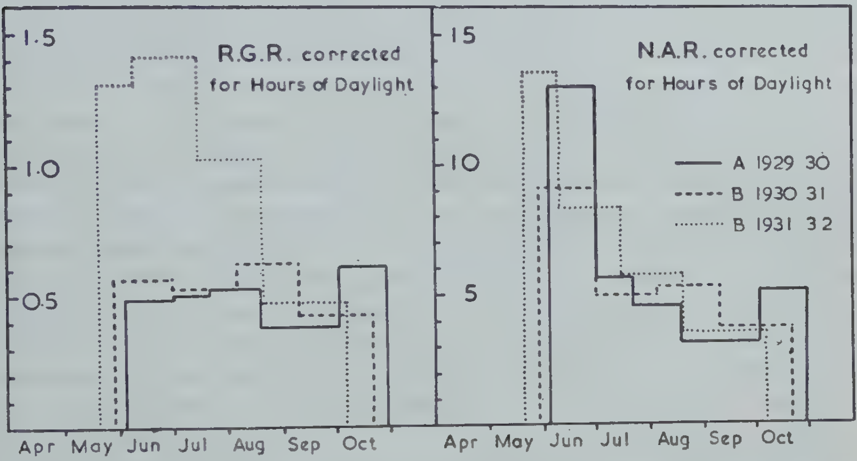

FIG. 7. *Correction for hours of daylight.* Adjusted values for the Relative Growth Rates (from Fig. 4, right) and Net Assimilation Rates (from Fig. 6, right) both corrected for the mean length of day—sunrise to sunset—for successive periods.

DISCUSSION

The values of the N.A.R., though lower than the maximum values quoted by Goodall (1945, p. 117), are not exceptionally low; indeed they are very like those found for cotton in the Sudan by Crowther (1944) and higher than those for cotton in Egypt. In general, the very low values for the R.G.R. found here are associated with low values of the leaf-weight ratio rather than with low values of the N.A.R., but, within each season, the values of R.G.R. and N.A.R. followed the same trend, both tending to fall as the season advanced, while the leaf-weight ratios tended to rise.

A very similar relative rate of growth has been reported for this same rootstock variety by Berry (1938) and rather lower rates for two other varieties. Rather higher values have sometimes been reported (Vyvyan, 1955), but there the trees were grown in rich soil that had recently been a hop garden. Low values of the R.G.R. seem rather characteristic of woody plants (Monselise, 1951), at any rate in the second season from the bud.

It is possible that both the low leaf-weight ratios and the low values of the R.G.R. in such plants may be due mainly to the presence of a large amount of residual tissue brought over from the previous season. In the first year of growth from a seed, or from a bud on a stool, there is no such mass of residual tissue and the relative rate of growth is no lower than that found in many seedlings. The seed, say 3 to a gramme, is of an average size, and the 1-year-old seedling at least as large as an average annual; the bud on the stool is about the size of a seed and the resulting rooted stem about that of a seedling. Nor is there any reason to suppose that the leaf-weight ratios of apple seedlings or budlings differ essentially in magnitude, or behaviour, from those of annual or herbaceous varieties.

The initial low values of the leaf-weight ratio are clearly due to the large amount of residual tissue brought over from the previous season; this is included in the total weight but not in that of the leaves, which start each season as nil. The relation of the tree to this residual tissue is somewhat analogous to that of a young potato plant to the parent tuber. This tuber is external to the young plant and ultimately withers away; its weight is not included when the leaf-weight ratio of the young plant is estimated. The residual tissues, however, become completely enclosed by the new tissues of the young tree and persist even where they do not all remain alive; their weight thus forms part of the total weight of the tree. It is possible, therefore that leaf-weight/(total-increment) rather than leaf-weight/(total-weight) would be the appropriate ratio to compare with the leaf-weight ratio of a young plant such as a potato. In this connexion it is of interest to note that Knight (1934) has shown that the annual increment in root weight in a young apple-tree is a constant proportion of the total increment in weight rather than of the total weight.

The low Relative Growth Rates found here, and reported for other woody plants, may likewise be due largely to the presence of the residual tissue. Some of the increase in tree weight may represent the enlargement or increase in weight of cells already present, but most will be due to the formation of new cells and tissues by cell-division. Some of the cortical cells of the residual tissues may divide, but few, if any, cell-divisions are likely to occur normally in the region bounded by the cambial zone, though the cells here, if alive, may retain the power to divide under special conditions. Most of the increment in tree-weight will thus be due to cell-division in the meristems—cambium and growing points—present in the residual tissue. These meristems may be regarded as the productive capital, the non-meristematic tissue as non-productive capital; the presence of a large amount of non-meristematic tissue will thus reduce the apparent relative rate of growth of the tree as a whole.

Although it is thus evident that the presence of the residual tissues is an important factor in the lowering both of the leaf-weight ratio and the Relative Growth Rate, there is no *a priori* reason for assuming that it is the only factor in either, or even the most important. The ratios will be the result of the factors determining the distribution of assimilates between the various organs and these will presumably be bound up with the physiological functions and performance of these organs. If the functions are reciprocal and each organ supplies the other with something essential for growth, the sizes and rates of growth will tend to be correlated positively. If they are in competition, the correlations may be negative. The old potato tuber apparently supplies the young plant as a whole with food material without determining its distribution and therefore the ratio between its organs. The residual tissue in the tree may function thus at first, but it persists, remains largely alive, and continues to some extent to perform other functions, such as translocation of water and solutes; it is possible that it may thus have a direct effect on the leaf-weight and other ratios.

Again, although the R.G.R., in so far as this represents cell-formation, will be influenced by the initial ratio of meristematic to non-meristematic cells, it will also be influenced by the average number of cells arising from each cell of the meristem. The meristems are made up of the cambium and the growing points, or buds, and these are essentially similar to the stool-bud from which the young tree arose, though they will vary in size. Most, if not all, these buds have the power to produce shoots as large as the original 1-year-old tree; indeed, under artificial conditions of extreme vigour they can produce something much larger than is normally found, as Maggs (1957) has shown. Few buds, however, produce such shoots; many form small spurs and others remain dormant. The fate of each bud, and the amount it grows, is determined largely by its distance from the pruning cut. The low R.G.R. is therefore due in part to the failure of the tree to produce as many cells per bud as were produced by the stool-bud from which it arose.

The data from these trees and from other material have been analysed in greater detail to provide fuller information on these problems and the results will be described and discussed in further papers in this series.

SUMMARY

1. Two series (A and B) of 1-year-old rooted-stems of the apple rootstock variety M.XIII were planted in consecutive seasons and samples were harvested at intervals over periods of 1 (A) and 2 (B) years respectively. Series A were planted unpruned but with roots removed, Series B were pruned but had their roots left on.

2. The weights at lifting and their logarithms were adjusted by covariance with the initial weights and their logarithms; this greatly reduced the S.E.s but changed the mean values very little.

3. There was an initial loss in total dry weight at the start of the season, comparable to that found in germinating seedlings.

4. The leaf-weight ratios were very low, they rose by August to about 14 per cent. in the first season after planting, and to about 20 per cent. in the second.

5. In the first season of both series the Relative Growth Rates started low—probably because of a check at planting—and remained low. In the second season (of Series B) the values started rather higher and then declined.

6. The Relative Leaf Growth Rates declined rapidly from an initial maximum. On the Arithmetic Mean basis, trees in their first year after planting behaved alike, but on the Geometric Mean basis trees unpruned at the start of the season—Series A and the second season of Series B—behaved alike. As the scatter was intermediate between normal and lognormal, neither basis was more legitimate than the other.

7. There were significant differences between the R.L.G.R.s and the R.G.R.s, and therefore significant changes in the ratio of leaf to total weight, during each interval in Series B, but in Series A, owing to high S.E.S., only that for the first period was significant.

8. The Net Assimilation Rates, on the weight basis, were not exceptionally low. They showed the same pattern as the R.L.G.R.s with rather less seasonal decline. Correction for day-length did not abolish the decline.

9. The low values of leaf-weight ratio and the R.G.R. were probably due mainly to the large initial mass of residual tissues. It is suggested that these are somewhat analogous to the old tuber in a young potato plant and that it is the leaf/total increment rather than the leaf/total weight that should be compared with the leaf-weight ratio in a young potato plant or a seedling.

There is evidence that other factors are involved.

ACKNOWLEDGEMENTS

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Growth Substances in Relation to the Rooting of *Salix fragilis* Cuttings

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ABSTRACT

An attempt has been made to correlate seasonal variations in the rooting responses of *Salix fragilis* cuttings with their content of growth-promoting substances. Although they were both low in early dormancy and higher in late dormancy and in summer there was not an exact correlation in all months.

It was found that extracts of *S. fragilis* cuttings taken in dormancy strongly inhibited the action of IAA on the growth of *Avena* coleoptile segments. This inhibitory influence was less in extracts of cuttings taken at the end of dormancy and in summer. Extracts of cuttings which had been left in water to root showed a striking decrease in their inhibitory action on IAA.

INTRODUCTION

ALTHOUGH it has been known for a very long time that woody cuttings show seasonal variations in their rooting responses, no attempts appear to have been made to correlate these with variations in their content of growth-promoting substances. Experiments were performed in Newcastle from 1949 to 1951 in which extracts were made of the growth substances in cuttings of *Salix fragilis* and an attempt made to correlate the growth-promoting or growth-retarding action of the extracts with the rooting capacity of the cuttings.

Van der Lek (1925) had previously demonstrated that the rooting capacity of cuttings of *Salix* spp. was high in spring when buds were sprouting and low in winter when buds were dormant. Gumpelmayer (1949) found that the seasonal variations in rooting responses of *S. fragilis* cuttings were more complex; the maximum number of roots were produced in the months of August, December, and April, and the minimum in the months of October, November, February, and in May.

The seasonal variations in the rooting responses of the *S. fragilis* material available at Newcastle were determined and are described under Materials and Methods. The assays of the growth-promoting substances present in extracts of *S. fragilis* cuttings during different months of the year are reported in Experimental Results (i).

Further experiments were performed to determine the changes in amounts and in distribution of growth-promoting and growth-inhibiting substances in cuttings during the time they were left in water to form roots. These experiments are described in Experimental Results (ii).

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MATERIALS AND METHODS

(i) Salix fragilis *material*

Initial experiments were performed to determine the rooting responses of the material available at Newcastle.

A number of selected trees were used as a source of material. Cuttings of the current year's growth consisting of the six terminal nodes and of medium length from 9·3 to 10·6 cm. were placed in tap water to a depth of 6 cm. and kept under a Perspex cover in a shaded part of the greenhouse. A high humidity was maintained within the cover by spreading over the base small pebbles which were kept constantly wet.

The water in which cuttings were standing was changed every third day. This was effective in preventing the growth of micro-organisms.

Series of 30 cuttings were set up every month. In the months of November 1949, March 1950, June 1950, and July 1950 two series were set up. Before June 1950 all cuttings were prepared from the growth of the season 1949. In June 1950 the first cuttings of the 1950 growth were employed.

The buds were dormant on all the cuttings taken from October 1949 to February 1949 and on all the cuttings of the 1950 growth. The cuttings taken in March bore swollen buds; the buds were bursting in April. In May and June the cuttings bore young sprouts.

The number of roots produced after 1 month is recorded in Table I.

TABLE I

Rooting in Salix fragilis *Cuttings throughout the Year*

IA. *Rooting in cuttings of the 1949 growth from October 1949 to June 1950.*
IB *Rooting in cuttings of the 1950 growth from June to October 1950.*

Month	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Sept.	Oct.
A. Average number of roots per 1949 cutting	2·75	(i) 1·33 (ii) 1·69	1·46	7·59	9·52	(i) 7·10 (ii) 7·47	6·17	10·79	(i) 13·38 (ii) 10·04	—	—	—
B. Average number of roots per 1950 cutting	—	—	—	—	—	—	—	—	3·92	(i) 6·07 (ii) 7·10	1·52	1·74

The buds remained dormant throughout the experiment on cuttings of the October, November, and December series using 1949 growth and on all cuttings of the 1950 growth. Sprouting of buds occurred after 15–20 days in the January and February series and after 4–5 days in the March and April series.

In these experiments rooting responses were low in the months of October, November, and December 1949. In January they were high and they remained high until June in which month they were at a maximum. Using the 1950 growth rooting responses were low in June and July and decreased further in September and October.

These experiments indicated that rooting responses were low during the months when the buds remained dormant, and high when the buds were sprouting. These results are in agreement with those of Van der Lek (1929). They contrast with Gumpelmayer's results (1948). It is possible that this can be attributed to a difference in the rooting medium; Gumpelmayer placed cuttings in a mixture of sand and peat, whilst in the Newcastle experiments cuttings were placed in water.

(ii) *Extraction of growth substances from* Salix fragilis *cuttings*

To extract growth substances from cuttings peroxide-free ether was used. Cuttings of standard length were dried of surface moisture, cut up into small pieces, and crushed in an electrical blendor in ether for 1–2 mins. After washing the blendor 3–4 times with ether all the extracts were collected and the ether decanted off. Three further half-hourly extractions were made with ether at 6° C. Van Overbeek, Olivo, and de Vasquez (1945) considered that all free auxin was extracted in two or three half-hourly periods and that growth-promoting substances extracted during a prolonged ether extraction could be attributed to the formation of free growth-promoting substances from a precursor during extraction. In a few experiments prolonged ether extractions were made during the following 21 hours using three lots of ether.

All the ether portions were collected and the ether distilled off. The residue was extracted with 100 c.c. of distilled water for 10 minutes.

(iii) *The assay of growth substances in extracts from* Salix fragilis *cuttings*

To assay the activity of extracts their effect was studied on the straight growth of *Avena* coleoptile segments. *Siegeshafer* Oats were supplied by the Swedish Seed Association. The grains were dehusked and germinated in an incubator in red light at 24°–25° C. and humidity 90 per cent. At 67 hours after sowing the coleoptiles had an average length of 1·5 cm. and were ready for sectioning.

All further operations were carried out in red light. Segments were cut of length 9·7 cm. at a distance of exactly 3 mm. from the tip of coleoptiles. The primary leaf was withdrawn and the segments mounted on glass threads. Twelve segments were placed in 40 c.c. of the solutions to be tested and solutions were aerated by a slow stream of moist sterile air.

Measurements of the length of segments were taken at 4-hourly intervals during the day in red light; intervals of 12 hours elapsed during the night without measurements. The final measurement was made after 45 hours.

(iv) *Significance of results*

Fisher's 't' test was used to determine the significance of the differences between means at their final length (Tippett, 1941). A 'significant difference' in this paper indicates that the difference between final mean lengths was significant at the 1 per cent. probability level.

The increases in length of segments were expressed as percentages of their original length.

GROWTH SUBSTANCES IN CUTTINGS OF *SALIX FRAGILIS* IN DIFFERENT MONTHS OF THE YEAR

Extractions were made of the growth substances present in *S. fragilis* cuttings in early and late dormancy and in summer. These are reported below under A, B, and C respectively.

(*a*) *Extractable growth substances in early dormancy*

Six extractions were made of dormant cuttings in December, January, and February. In all experiments extractions were made in the first three half-hours (extract 1) and in the following 21 hours (extract 2).

In Experiment 1 30 cuttings were extracted and in Experiment 2 the number of cuttings was increased to 60 because it was probable that growth-promoting substances were present in very low concentrations at this time of the year and would not be demonstrable except by using larger quantities of material. The results of Experiments 1 and 2 can be seen in Table II.

TABLE II

Percentage Increase in Length of Coleoptile Segments in Distilled Water, IAA, and Extracts of Salix fragilis *Cuttings in December* (X_1 *and* X_2)

Expt. no.	Percentage increase of length in coleoptile segments in:						
	DW	IAA	X_1	X_1 1:10	X_1 1:100	X_2	X_2 1:10
1	17·6	99·4	29·2	19·6	—	15·4	60·4
2	16·9	93·3	7·1	31·7	22·3	—	—

DW: distilled water.
IAA: 1 mg./litre IAA.
X_1: Extract 1 (see text).
X_2: Extract 2 (see text).
X_1 1:10 : X_1 diluted one part in ten.
X_1 1:100: X_1 diluted one part in a hundred.
X_2 1:10 : X_2 diluted one part in ten.

In Experiment 1 the increase in X_1 was significantly greater than that in DW. There was no significance in the differences between final increases in DW and X_1 1:10. Thus there was evidence of the presence of only small amounts of growth-promoting substances in X_1. The presence of growth-promoting substances in X_2 is clearly indicated by the increase in the dilution 1:10 which was considerably greater than that in DW. However, in undiluted X_2 the final increase was less than in DW, thus it is possible that in X_2 there was present a supra-optimal concentration of growth-promoting substances.

In Experiment 2 the final increase in X_1 was significantly less than that in DW. The final increases in X_1 1:10 and X_1 1:100 were significantly greater than that in X_1 and that in DW. Because coleoptile growth was greater in the dilutions it may be possible that in X_1 there was present a supra-optimal concentration of growth-promoting substances. However, the increases in X_1 1:10 and in X_1 1:100 were not notably large (cf. increase in IAA) and it can be assumed that the growth-promoting activity of X_1 was not very great.

At this stage the question arose as to whether these first extracts which showed no, or very little, growth-promoting activity, had any inhibitory action on IAA. If this were the case a 1 mg./litre IAA solution made up in these extracts would have less growth-promoting activity than a 1 mg./litre IAA solution made up in water. Four experiments were performed in which the action of 1 mg./litre IAA made up in these extracts was investigated.

In all four experiments 60 cuttings were extracted. The results of Experiments 3, 4, 5, and 6 are shown in Table III.

TABLE III

Effects of Extracts made from Dormant Cuttings in January and February on the Percentage Increase in Length of Coleoptile Segments

(See heading to Table II)

Expt. no.	Percentage increase in length of coleoptile segments in:					
	DW	IAA	X_1	X_1+IAA	X_2	X_2+IAA
3	25·4	113·3	15·9	33·4	5·9	—
4	24·8	106·1	45·2	83·9	41·8	—
5	28·6	99·1	24·9	54·1	30·1	80·1
6	26·9	108·4	13·2*	16·9	45·6	89·7

* This value represents the percentage increase after 33 hours.
X_1+IAA: 1 mg./litre IAA made up in X_1.
X_2+IAA: 1 mg./litre IAA made up in X_2.
For other abbreviations see Table II.

In Experiments 3 and 5 the increases in X_1 were less than those in the corresponding DW series, the difference being significant in Experiment 3. In Experiment 6 the increase in X_1 was 13·2 per cent. after 33 hours and was significantly less than that of 25·1 per cent. in DW after the same time. From these three experiments there was no evidence of the presence of growth-promoting substances in X_1.

In Experiments 3, 5, and 6 final increases in X_1+IAA were substantially less than the corresponding increases in IAA alone. Thus X_1 showed a strong inhibitory effect on the action of IAA.

The results of Experiment 4 are rather different from those of the other experiments. The final increases in X_1 were significantly greater than in DW and in X_1+IAA were significantly less than in IAA alone. Apparently X_1 had growth-promoting activity and at the same time inhibited the action of IAA. The problem of the effect of such solutions will be considered under Discussion.

In Experiments 4, 5, and 6 the final increases in X_2 were greater than those in the corresponding DW cultures although the difference was not statistically significant in Experiment 5. In Experiment 3 the increase in X_2 was much less than that in DW. It is noteworthy that after 6 hours the increase in X_2 was 15·0 per cent. which was followed by shrinkage after 10 hours. A high initial growth-rate followed by shrinkage often occurs in very high concentrations of auxin and it is not impossible that there was present in the solution a supra-optimal concentration of growth-promoting substances. This suggestion could only be confirmed had series been set up in dilutions of X_2.

In Experiments 5 and 6 coleoptile segments showed greater increases in X_2+IAA than in X_1+IAA. Apparently X_2 exerts less inhibition on the action of IAA than X_1.

The results of Experiments 1–6 suggest that very small amounts of growth-promoting substances were present in X_1 from dormant *S. fragilis* cuttings. The presence of considerable amounts found after prolonged ether extraction could probably be attributed to the formation of free growth-promoting substances from a precursor during extraction. Considerable amounts of a substance which inhibited the action of IAA on coleoptile extension could be extracted from dormant *S. fragilis* cuttings in the first three half-hours. Smaller amounts of this substance were obtained by a prolonged extraction. The inhibitor acted in these experiments not by itself inhibiting growth but rather by an effect on IAA.

(*b*) *Extractable growth substances in late dormancy*

Four extractions were made of cuttings in March and early April. In 1951 the buds on *S. fragilis* trees began to swell and to burst from April 3 to April 10.

Sixty cuttings were extracted in each experiment. Extracts were made of buds and stems separately in an attempt to localize the growth substances. Only the first three half-hourly extractions of each were made. The results of Experiments 7, 8, 9, and 10 are shown in Table IV.

TABLE IV

Effects of Extracts made from Cuttings in March and early April on the Percentage Increase in Length of Coleoptile Segments

(*See heading to Table II*)

Expt. no.	Percentage increase in length of coleoptile segments in:					
	DW	IAA	XS	XS+IAA	XB	XB+IAA
7	33·1	102·2	23·4	32·2	60·9	90·5
8	23·8	94·0	15·9	23·0	70·7	81·3
9	23·0	93·4	54·7	79·2	40·5	91·3
10	27·2	108·1	50·0	46·9	62·9	83·8

XS: Extracts of stems. XB: Extract of buds.
XS+IAA: 1 mg./litre IAA in XS. XB+IAA: 1 mg./litre IAA in XB.
For other abbreviations see Table II.

In Experiments 7 and 8 segments in XS showed increases which were significantly less than those in the corresponding DW series. XS appeared to have a small growth-inhibitory activity in these experiments. The increases in XS+IAA were not significantly different from those in DW, indicating the possible presence of large amounts of growth-inhibiting substances in XS. In XB increases were considerably higher than in DW, indicating the presence of growth-promoting substances. In XB+IAA the measured final increases were less than those in IAA alone. The difference was statistically significant in Experiment 8. This suggests that either some inhibitory substances were present in XB or that in XB+IAA there was a supra-optimal concentration of growth-promoting substances causing less growth than in IAA alone. The problem of such extracts which themselves have growth-promoting activity and also inhibit the action of IAA will be considered in the Discussion.

In Experiments 9 and 10 coleoptile segments showed increases in XS that were significantly greater than those in DW, indicating the presence of growth-promoting substances in XS. In XS+IAA increases were significantly less than in IAA alone, again indicating that XS had a growth-inhibitory activity. The final increases in XB were significantly greater than in DW, indicating that XB continued growth-promoting substances. In both experiments there was less elongation in XB+IAA than in IAA alone, although the difference was only significant in Experiment 10. XB in these experiments had growth-promoting activity and also inhibited the action of IAA. The problem of the effects of such extracts is considered in the Discussion.

All four experiments on cuttings in late dormancy indicated that growth-promoting substances were present in buds. At the same time the extract of buds inhibited the action of IAA. The problem of the effects of such extracts is considered in the Discussion.

No evidence was obtained of the presence of growth-promoting substances in the extracts of stems in the earlier experiments. It is possible that the presence of growth-promoting substances was masked by inhibitory substances which were shown to be present in very large amounts. In the later experiments the extracts of stems had a growth-promoting activity and they also inhibited the action of IAA although to a smaller extent than in the earlier experiments.

(*c*) *Extractable growth substances during summer*

Three assays (see Table V) were similarly made on extracts of cuttings in June and July. The sprouts on the cuttings were very large at the time of the summer experiments, so in order to keep material within manageable amounts only one-fifth of the total number of buds was extracted.

TABLE V

Effects of Extracts made from Cuttings in Summer on the Percentage Increase in Length of Coleoptile Segments

(*See heading to Table II*)

Expt. no.	Percentage increase in length of coleoptile segments in: DW	IAA	XS	XS+IAA	XB	XB+IAA
11	41·9	114·4	57·2	80·2	57·5	70·8
12	42·5	96·9	64·7	70·3	50·0	78·8
13	30·6	115·0	37·5	57·7	50·9	72·4

Abbreviations: see Tables II and IV.

In all three experiments the final increases both in XS and in XB were significantly greater than the corresponding increases in DW. In both XS+IAA and in XB+IAA the final increases were less than in the corresponding series in IAA alone.

Experiments 11, 12, and 13 suggest that growth-promoting substances were present in the extracts of stems and buds in summer. These extracts have, however, an inhibiting effect on IAA action, suggesting either the presence of inhibitors or of a supra-optimal concentration of growth-promoting substances. The problem of the effects of such extracts is considered further in the Discussion.

If the results of experiments in early and late dormancy and in summer are considered together it seems that in early dormancy very small amounts of growth-promoting substances were present. Another substance was present which had very little effect on the growth of coleoptile segments, but which had a strongly inhibitory influence on the action of IAA. In late dormancy growth-promoting substances first became evident in buds and later in stems. At this time extracts of buds and stems had an inhibitory effect on the action of IAA, but this effect was smaller than in early dormancy. This suggests either that growth-inhibitory substances were present in buds and stems at this time or that a supra-optimal concentration of growth-promoting substances was present in the mixture of the extracts and IAA (see Discussion). In summer growth-promoting substances were present in sprouts and stems and again the extracts of the stems and buds inhibited the action of IAA (see Discussion).

CHANGES IN AMOUNTS AND DISTRIBUTION OF GROWTH SUBSTANCES IN CUTTINGS OF *S. FRAGILIS* DURING ROOTING

Two series of experiments were performed to study changes in amounts and distribution of growth substances during the time that cuttings were placed in water to form roots; in the first (see A below) a comparison was made of growth substances in cuttings direct from the tree with those from cuttings left to root, and in the second (see B below) assays were made of the apical and basal halves of cuttings which had been left to root for 1 week and for 1 month.

(*a*) *Comparison of growth-promoting substances present in extracts from cuttings direct from the tree and from cuttings rooted for 5 days*

Two experiments (14 and 15) were performed in March and in April. In each experiment 120 cuttings were collected and 60 of these were extracted immediately. The remaining 60 were left to root in water for 5 days and were extracted on the 6th day.

The result of Experiments 14 (*a* and *b*) and 15 (*a* and *b*) are shown in Table VI.

In Experiment 14 elongations in XS from cuttings direct from the tree were less than in the corresponding DW series; elongations in XS from cuttings rooted for a week were greater than in the corresponding DW series. Increases of segments in XS+IAA from cuttings direct from the tree were similar to those in DW. In XS+IAA from the rooted cuttings elongations were significantly greater than in the DW series.

In Experiment 14 the final increases in XB from cuttings direct from the tree were significantly greater than those in the corresponding DW series.

The increases in XB from cuttings left to root were not significantly greater than in DW. In XB+IAA from cuttings direct from the tree the increases were less than in IAA alone. In XB+IAA from cuttings left to root the increase was also less than in IAA alone, the difference being greater than in extracts from cuttings direct from the tree.

In Experiment 15 similar results were obtained. Both in cuttings direct from the tree and in cuttings left to root elongations were greater in XS than in the corresponding DW, but the difference was greater in the latter. In XS+IAA both in cuttings direct from the tree and in cuttings left to root elongations were less than in IAA alone, but the difference was smaller in extracts from cuttings left to root.

TABLE VI

Effects of Extracts made from Cuttings Direct from the Tree (Expts. 14a and 15a) and Extracts from Cuttings Rooted for 5 Days (Expts. 14b and 15b) on the Percentage Increase in Length of Coleoptile Segments

(See heading to Table II)

Expt. no.	Percentage increase in length of coleoptile segments in:					
	DW	IAA	XS	XS+IAA	XB	XB+IAA
14*a*	23·8	94·0	15·9	23·0	70·7	81·3
14*b*	30·7	103·5	44·5	55·6	33·6	42·4
15*a*	27·2	108·1	50·0	46·9	62·9	83·8
15*b*	30·5	110·6	74·9	91·7	37·1	65·6

Abbreviations: see Tables II and IV.

In Experiment 15 there was a larger difference between elongations in DW and in XB in cuttings taken direct from the tree than in cuttings left to root. There was a larger difference between elongations in XB+IAA and IAA in cuttings left to root than in cuttings direct from the tree.

It is difficult to compare the activity of extracts on different occasions, but these two experiments would seem to indicate that stems of cuttings of *S. fragilis* left in water for 5 days contained more growth-promoting substances than those direct from the tree. During these 5 days the growth-promoting substances present in the buds appear to have decreased. It seems that the inhibiting effect of stem extracts decreased and the inhibiting effect of bud extracts increased during this time.

In Experiments 14 and 15 there are a number of extracts which themselves promote growth and also inhibit the action of IAA. Such extracts are XS in Experiments 14*b* and 15*a* and *b*, and XB in Experiments 14*a* and *b* and 15*a* and *b*. For a consideration of the problem of the effects of such extracts see Discussion.

(*b*) *Comparison of the growth substances present in apical and basal halves of cuttings which had been left to root*

Two experiments were performed in May and June on cuttings which had been allowed to root for (i) 1 week (ii) 1 month. After 7 days the cuttings were swollen at the base and in a few cases short roots were emerging. At 1 month the cuttings had copious roots and had become virtually independent established plants.

In all assays buds and roots were removed and extracts were made only of the stems. Small cuttings were used in each assay, each cutting being cut into two equal pieces which were assayed separately.

The results of Experiments 16, 17, 18, and 19 are shown in Table VII.

TABLE VII

Effects of Extracts from Cuttings left to Root for Seven Days (Expts. 16 and 17) and Extracts from Cuttings left to Root for one Month (Expts. 18 and 19) on the Percentage Increase in Length of Coleoptile Segments

(*See heading to Table II*)

Expt. no.	Percentage increase in length of coleoptile segments in:					
	DW	IAA	XBa	XBa+IAA	XA	XA+IAA
(i) 16	35·7	105·4	70·7	94·6	55·6	106·6
17	43·7	106·4	70·0	86·0	65·7	81·4
(ii) 18	37·8	103·0	61·6	92·2	67·8	107·2
19	42·6	110·2	56·0	92·4	88·7	112·8

XBa: Extract of basal halves of cuttings.
XBa+IAA: 1 mg./litre IAA in XBa.
XA: Extract of apical halves of cuttings.
XA+IAA: 1 mg./litre IAA in XA.
For other abbreviations see Table II.

In Experiments 16 and 17 the final increases in XBa were substantially greater than the corresponding increases in DW and greater than the corresponding increases in XA. The difference between elongations in the corresponding XBa and XA series were statistically significant at the 1 per cent. probability level in Experiment 16 and at the 5 per cent. probability level in Experiment 17.

In Experiment 16 the increase in XBa+IAA was a little less than in IAA alone and the increase in XA+IAA was a little more than in IAA alone, but neither of these differences was significant. In Experiment 17 the increases in XA+IAA and in XBa+IAA were both significantly less than that in IAA alone. Both in Experiments 16 and 17 the inhibitory influences on IAA of XA and XBa were notably smaller than in earlier experiments.

Assays of extracts from cuttings which had been left to root for 7 days indicated that more growth-promoting substances were present in the basal parts than in the apical parts. The inhibitory effects on IAA of the extracts of both parts were very small.

In both Experiments 18 and 19 on cuttings rooted for 1 month the increases in XA were greater than the increases in XBa. The difference was significant in Experiment 19. In both experiments elongations in XA and in XBa were significantly greater than in DW.

In Experiments 18 and 19 the final increases in XBa+IAA were significantly less than those in IAA and those in XA+IAA. Increases in XA+IAA were greater than those in IAA but the differences were not significant. The inhibitory effects of these extracts on IAA were strikingly less than those of extracts from stems in earlier experiments.

After one month when the cuttings were virtually independent established plants growth-promoting substances were evident throughout the cutting and were no longer present in larger amounts in the basal portions. Indeed the suggestion is that more growth-promoting substances were present in apical halves of cuttings, although the differences were not significant in both experiments. The inhibitory effects of IAA were very small in the extracts of basal parts and were absent altogether in the extracts of apical parts.

DISCUSSION

Although it was found that there was some correlation between seasonal variations in rooting responses and seasonal variations in the content of growth substances in *Salix fragilis* cuttings, this correlation was not exact throughout the year. It was found that in December, when rooting responses were low, a 3-hourly ether extraction yielded no growth-promoting substances. In January and February, when the rooting responses were considerably higher, there were still only small amounts of growth-promoting substances extractable in the first three half-hours and it was not therefore possible to correlate the higher rooting capacity with a higher concentration of free growth-promoting substances (see Experiments 1–6).

From December to February a prolonged ether extraction of cuttings yielded considerable amounts of growth-promoting substances (see Experiments 1, 3, 4, 6). Van Overbeek *et al.* (1945) have indicated that all free auxin was extracted from sugar-cane tissue in the first three half-hours, and during a prolonged ether extraction auxin was being produced continuously from a precursor. If *S. fragilis* and sugar-cane materials are similar in this respect it can be assumed that growth-promoting substances present in prolonged ether extracts are being produced from a precursor. It is possible that amounts of auxin precursor present in cuttings could be correlated with the rooting responses, but no experiments were performed along these lines.

From December to February a substance was extracted from *S. fragilis* cuttings which strongly inhibited the action of IAA on coleoptile segments (see Experiments 3, 5, 6). This substance was mostly extracted in the first three half-hours although certain smaller amounts were obtained from the subsequent overnight extraction (see Experiments 5, 6). This inhibiting substance acted by not itself causing decreased elongation, but rather by inhibiting the elongation stimulated by IAA. Hemberg (1949) has shown that a substance which inhibited the action of IAA in the standard *Avena* curvature was present in *Fraxinus* buds in early dormancy and absent at the end of dormancy.

In January and February the buds on cuttings began to sprout after 15–20 days. Changes occurring before and after the sprouting of these buds would undoubtedly affect the auxin balance and distribution in the cuttings and influence their rooting responses.

During the months of March, April, June, and July growth-promoting substances become evident at first in the buds and later in the stems of cuttings taken direct from the tree (see Experiments 7–13). During these months the rooting responses were high. It has been shown previously that growth-promoting substances were present in swelling buds (Avery, Burkholder, and Creighton, 1937; Gunkel and Thimann, 1949) in bursting buds (Czaja, 1934; Soding, 1936; Zimmermann, 1936; Avery, Burkholder, and Creighton, 1937), and in young shoots (Avery, Burkholder, and Creighton, 1937).

Although extracts from buds and stems during late dormancy and in early summer had a considerable growth-promoting activity they also had an inhibitory effect on the action of IAA (see Experiments 7–13).

In many experiments there were a number of extracts which caused a stimulation of elongation of segments and at the same time had an inhibitory effect on the action of IAA on coleoptile growth. Such extracts were X_1 in Experiment 4, XB in Experiments 7–15, XS in Experiments 9–13, 14*b*, 15*a* and 15*b*, XBa in Experiments 17–19, and XA in Experiment 17. In these extracts there was presumably a mixture of growth-promoting and growth-inhibiting substances of which growth-promoting substances were present in greater quantity as the coleoptile segments in the extracts showed elongations greater than those in DW. Had the growth-promoting substance in the extract been IAA it could be assumed that the inhibitory substances were exhibiting their maximum effect against this IAA, and could exert no further effect against added IAA. However, in IAA made up in these extracts coleoptile segments elongated less than in IAA alone. It is possible that in the extracts together with IAA there was present a supra-optimal and toxic concentration of growth-promoting substances. The other possibility is that IAA and some other growth-promoting substance were present together in the extracts and that the inhibitory substance in the presence of both growth-promoting substances showed a preferential inhibition of the unknown one.

It was shown in Experiments 14–19 that the inhibitory influence of extracts on IAA decreased strikingly when cuttings were left in water to form roots. It has been suggested previously by Khan and Hall (1954) that inhibitory substances were of some importance in the rooting of cuttings. These authors found that sugar-cane cuttings that had been standing in water for 24 hours before planting showed increased rooting responses; it was suggested that during this time a substance inhibitory to rooting was leached into the water.

During the first 5 days that cuttings were left in water the growth-promoting activity increased in the stems and decreased in the buds (see Experiments 14, 15). This suggested that growth-promoting substances were discharged by the buds and accumulated in the stems. That they accumulated in the basal halves of cuttings is indicated by the fact that extracts of these portions had a higher growth-promoting activity than extracts of apical portions after 7 days (see Experiments 16, 17). After 1 month when the cutting was an established plant growth-promoting substances were no longer present in larger amounts in the basal portions (see Experiments 18, 19).

Changes in the amounts and distribution of growth-promoting substances during regeneration have been investigated by Brandes and van Overbeek (1948), Kulescha and Camus (1948), Warmke and Warmke (1950), and Hemberg (1951). These Newcastle results are in agreement with those of Kulescha and Camus (1948) and Warmke and Warmke (1950) who found increases in free auxin in the basal halves of cuttings of Endive and Chicory respectively during the first few days of regeneration. However, Brandes and van Overbeek (1948) using sugar-cane cuttings and Hemberg (1951) using *Phaseolus* cuttings were unable to detect any increase in free auxin during the first few days that cuttings were left to root although there was a considerable increase in bound auxin, extractable in 21 hours.

It was obviously desirable that the growth-promoting and growth-inhibiting substances present in these extracts of cuttings should be separated and, if possible, identified. Before this work was discontinued a few preliminary qualitative experiments were performed using paper partition chromatographic methods as outlined by Bennet-Clark, Tambiah, and Kefford (1952) (reported Tyce, 1952).

Using water-saturated butanol ammonia as solvent there was evidence of the presence in extracts of cuttings of a growth-promoting substance with an R_F value of 0·35–0·40 which was very close to the observed R_F for pure IAA. In one experiment only there was apparent a second growth-promoting substance of R_F 0·6–0·7 in the solvent used.

When the different regions of chromatograms were eluted in 10 mg./litre IAA an inhibiting substance of R_F 0·5–0·6 was found to be present. Inhibitory substances have been demonstrated in extracts by a number of workers (Luckwill, 1952; Bennet-Clark, Tambiah, and Kefford, 1952; Lexender, 1953; Blommaert, 1954) who found that the substance inhibited growth in water eluates. It is noteworthy that in the Newcastle experiments the inhibitory substance was not always evident in water eluates but became very evident in the presence of IAA.

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Isotopic Studies of Nitrogen Fixation in Non-Legume Root Nodules

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ABSTRACT

Isotopic studies are presented on six of the eight recognized non-legume nodule-forming genera of Angiosperms. Evidence has been obtained of the occurrence of fixation of atmospheric nitrogen in the root nodules of *Casuarina*, *Ceanothus*, and *Shepherdia*, now examined isotopically for the first time. In further studies of the nitrogen-fixing nodules of *Hippophaë* and *Alnus* it is shown that the fixation continues for a longer period after detachment from the plant than is the case with legume nodules, and that the enrichment in ^{15}N which they (and also detached *Casuarina* nodules) finally achieve after exposure to excess of the free isotope considerably exceeds that shown by detached legume nodules. Fixation in detached *Myrica* nodules was not clearly affected by reduction in oxygen supplied until the proportion of oxygen was less than 5 per cent.

INTRODUCTION

PREVIOUS reports (Bond, 1955; Bond and MacConnell, 1955) referred to isotopic studies of the fixation of atmospheric nitrogen associated with the root nodules of three non-legume genera (*Alnus*, *Myrica*, and *Hippophaë*) native to Britain and also of widespread occurrence in some other countries. In the present articles further observations are presented on these genera, together with data for three other genera of nodule-forming habit, namely *Casuarina*, *Shepherdia*, and *Ceanothus*. *Casuarina* is a prominent genus of tropical and sub-tropical regions, while *Shepherdia* is of frequent occurrence in Canada, extending sometimes into the Arctic Circle; data derived from long-term growth experiments indicative of the occurrence of fixation of nitrogen in the nodules of these genera have been provided by Bond (1957) and Gardner and Bond (1957). *Ceanothus* species are prominent members of the native flora in certain areas of the United States.

METHODS

Nodulated plants of the above genera were grown in water culture in a solution free of combined nitrogen by methods previously described (Bond, 1955; Bond, MacConnell, and McCallum, 1956). For the inoculation of *Casuarina* plants, nodules were obtained by air-mail from various centres abroad (see Bond, 1957, for detailed acknowledgements), while Dr. W. W. Wagener, California Forest and Range Experiment Station, U.S. Department of Agriculture, kindly sent nodules of *Ceanothus* for inoculation purposes. *Shepherdia* plants were inoculated from nodules of *Hippophaë*, which are probably inhabited by the same micro-organism.

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In tests involving whole plants, the nodulated root systems were exposed to gas mixtures (specified later) containing an excess of free ^{15}N by means of an experimental arrangement previously described (Bond, 1955). In those with detached (excised) nodules the latter were enclosed in stout specimen tubes during exposure to the isotope. In all cases the gas mixtures were supplied at atmospheric pressure. After exposure the plant material was prepared for mass spectrometric analysis as described by Bond and Scott (1955); the author is greatly indebted to Dr. A. S. McFarlane and Mr. G. Dickinson, of the National Institute for Medical Research, Mill Hill, for the analyses. Control samples, not exposed to excess ^{15}N, were included in the samples submitted for analysis, and all gave values very close to the normal figure (0·375 atom per cent.), the maximum deviation being 0·004 atom per cent. All the exposures of plant material to isotopic nitrogen described below were made in 1955, except in the case of detached *Casuarina* nodules, which were exposed in the following year.

DATA OBTAINED

A. *Experiments with whole plants*

Table I shows the results of an experiment with *Casuarina* plants. They present the same picture as previous data for other non-legumes and for legumes (Bond, 1955), with a marked enrichment shown in the nodules after exposure of the root system to excess ^{15}N, and a lower enrichment elsewhere. It is concluded that the nodule is the seat of the fixation, and that the products of fixation are fairly rapidly translocated to other parts of the plant. The marked increase in ^{15}N content of the shoots of plants 3 and 4 suggests that had it been possible to assay the nodules from each plant separately, particularly substantial enrichment would have been shown by the nodules of these plants.

TABLE I

Increase in Fixed ^{15}N in Plants of Casuarina cunninghamiana *after Exposure of Nodulated Root Systems to Excess Free ^{15}N*

	Increase in ^{15}N content, as atom per cent. of total nitrogen, over normal value		
Plant no.	Shoot	Roots	Nodules
1	0·601	0·286	1·464
2			
3	1·181		
4			
5	0·280		
6			

First-year plants with a mean shoot height of 17 cm. were used. The gas space in the bottle enclosing each root system was charged with a gas mixture containing 15 per cent. nitrogen (with 36 atom per cent. ^{15}N), 20 per cent. oxygen, and 65 per cent. argon. The period of exposure was 96 hours, in September. It was necessary to combine some samples to provide sufficient nitrogen for analysis.

Owing to the author's unfamiliarity at the time of these experiments with methods of breaking the dormancy of *Ceanothus* seeds, only two nodulated plants (both of *C. azureus*) were available. The plants showed a clear ability to grow in culture solution free of combined nitrogen, and confirmation of the nitrogen-fixing property thus indicated for the nodules was sought in an isotopic test on one of the plants in September. This was carried out as for *Casuarina* (Table I), the period of exposure being 72 hours. In order to provide sufficient nitrogen for assay it was necessary to combine the nodule and root material. This combined sample showed an enrichment of 0·342 atom per cent., and the shoot one of 0·047 atom per cent. Thus there is clear evidence that fixation is associated with the root system, and it is reasonable to attribute it to the nodules by analogy with other genera.

The root systems of two nodulated plants of *Shepherdia canadensis* were similarly exposed to excess free ^{15}N for 72 hours. The nodules, combined with some root material for assay, showed as a result an enrichment of 0·216 atom per cent., proof of the occurrence of fixation. Roots alone showed an enrichment of 0·083 and shoots of 0·045 atom per cent.

B. *Experiments with detached nodules*

It has been demonstrated previously that *Alnus* and *Myrica* nodules show a partial retention of nitrogen-fixing powers after separation from the plant. In presenting the results of further studies on detached nodules, it is emphasized that the ^{15}N data provided are atom per cent. of the whole of the nodule nitrogen.

Fig. 1 shows the progress with time of the fixation in detached nodules of *Alnus* and *Hippophaë*. In *Alnus* appreciable fixation is shown until about 12 hours from detachment, but thereafter there is little further fixation. In the *Hippophaë* nodules fixation was longer-lived, and a very substantial final enrichment (0·645 atom per cent.) was achieved by these nodules. Also notable in these experiments is the close agreement between duplicate samples, pointing to a uniformity of material.

Table II shows the results of tests with detached nodules of *Casuarina*, very substantial enrichment being indicated. With *Shepherdia* the amount of nodules available was too small for them to be treated separately, and detached roots bearing nodules were exposed to excess ^{15}N, other samples of nodule-free roots from the same plants being similarly exposed as control material. The results are given in Table III. On both testing occasions clear evidence of nitrogen-fixing ability was obtained for the nodulated roots; the slight enrichment shown by one of the samples of designedly nodule-free roots was possibly due to the presence of very young nodules which are distinguished with difficulty from lateral roots.

Table IV presents the results of an experiment on the effect of oxygen supply on fixation in detached *Myrica* nodules. Fixation proved to be rather small and also variable from sample to sample, both effects being probably due to the use of nodule material from a 3-year-old plant. It is clear that

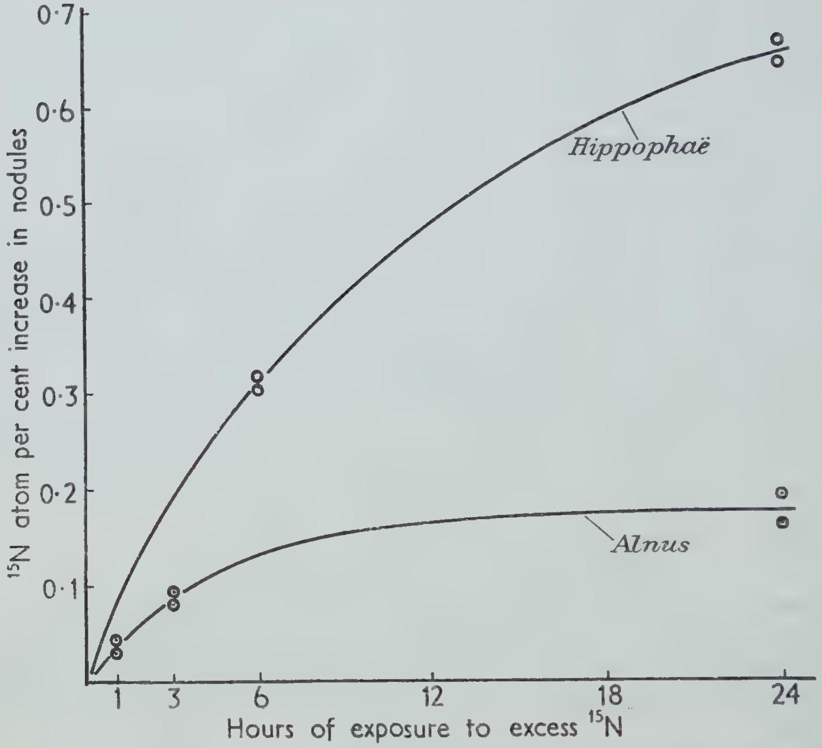

FIG. 1. Time course of the fixation in detached nodules of *Alnus glutinosa* and *Hippophaë rhamnoides.*

In the *Alnus* experiment the nodules were taken from 20 first-year plants in August, 0·8 g. samples (fresh weight) being enclosed in 25 ml. specimen tubes which were then charged with a gas mixture similar to that indicated in Table II. One hour elapsed between the commencement of nodule detachment and the simultaneous gassing of all the samples. The nodules were held at 24° C. during exposure. The mean total nitrogen per sample was 5·5 mg. With *Hippophaë* the nodules were taken from 24 first-year plants in August, and samples of 0·5 g. enclosed and exposed as above, except that the gas mixture contained 20 per cent. of nitrogen. The interval between start of nodule detachment and the gassing of all the samples was 25 minutes, and the mean total nitrogen content per sample was 2·9 mg. In both experiments there were two samples for each period of exposure.

TABLE II

Test for Fixation in Detached Nodules of Casuarina cunninghamiana

Sample	Increase in ^{15}N content, as atom per cent. of total nitrogen, over normal value
1	0·336
2	0·274
3	0·368
4	0·279

The samples, each of 1·5 g. fresh weight, of nodules and nodule-roots, from second-year plants, were enclosed in 25 ml. specimen tubes which were then charged with 10 per cent. nitrogen (containing 36 atom per cent. ^{15}N), 20 per cent. oxygen, and 70 per cent. argon. The interval between the commencement of nodule detachment and the simultaneous gassing of all the samples was 15 minutes. The samples were exposed to the gas mixture for 19 hours at 24° C. in August; the mean total nitrogen content per sample was 6·4 mg.

TABLE III

Tests for Fixation in Shepherdia canadensis

Type of sample	Increase in ^{15}N content, as atom per cent. of total nitrogen, over normal value: August test	September test
Roots bearing nodules	0·037	0·153
Roots without nodules	0·011	nil

The material, taken from first-year plants, was enclosed together with 4 ml. culture solution in 25 ml. tubes which were then charged with a similar gas mixture to that indicated in Table II. The exposure was continued for 24 hours at 20° C.

TABLE IV

Effect of Oxygen Tension on Fixation in Detached Nodules of Myrica gale

Sample	Initial proportion of oxygen provided	Increase in ^{15}N content, as atom per cent. of total nitrogen, over normal value
1	20%	0·058
2		0·021
3	12%	0·061
4		0·030
5	5%	0·012
6		0·052
7	1%	nil
8		nil

Samples of nodules and nodule-roots of 1·5 g. fresh weight were taken from a 3-year-old plant and enclosed in 25 ml. specimen tubes which were then charged with gas mixtures containing 10 per cent. nitrogen (with 36 atom per cent. ^{15}N), oxygen as indicated above, and argon to 100 per cent. The interval between the commencement of nodule detachment and the gassing of the samples was about 1 hour. The exposure to the gas mixtures was continued for 4 hours at 23° C., in August. Mean total nitrogen content per sample = 2·9 mg.

fixation was suppressed at an oxygen level between 5 and 1 per cent., but over the range of 20 to 5 per cent. no oxygen effect on fixation is discernible.

DISCUSSION

The occurrence of a vigorous fixation of nitrogen in the nodules of the non-legume genera *Alnus*, *Myrica*, and *Hippophaë* had been established by previous work (Bond, Fletcher, and Ferguson, 1954; Bond, 1956; also references cited earlier in the present article). The isotopic data now provided indicate that fixation likewise occurs in the nodules of *Casuarina*, *Shepherdia*, and *Ceanothus*. In respect of the first two of these genera the new results confirm the conclusions drawn from long-term growth experiments (Bond, 1957; Gardner and Bond, 1957). The data now provided for *Ceanothus* constitute, so far as the author is aware, the first published direct evidence for fixation in the nodules of that genus, though many writers have conjectured that the nodules might be so active. Snyder (1925) claimed that a colleague had isolated the causal organism from *Ceanothus* nodules and had obtained evidence of nitrogen-fixing properties, but no details were provided and no further publication relating to this work has been traced. Quick (1944) recorded pot culture data which appeared to point to a soil-improving effect of *Ceanothus*, but he himself did not express any conclusion as to their explanation. *Ceanothus* species were described by Wahlenburg (1930) as becoming specially prominent in forest areas devastated by fire, and his conjectures as to the possible value of the genus in restoring soil nitrogen under such circumstances receive some substantiation from the data now presented. It is true that the data are based on two plants only, but the possession or lack of nitrogen-fixing properties are not characters in which variation between individual plants of a species or genus is to be anticipated, setting aside the possible occurrence of 'ineffective' nodules, of which there is as yet no evidence in other non-legume genera.

The original tests by the Madison workers (see below for references) for the occurrence of fixation in detached legume nodules were undertaken partly in search of final proof that the nodules are responsible for the fixation associated with legumes, and also in the knowledge that if the detached nodules proved to continue fixing nitrogen on a scale measurable isotopically, then the investigation of various aspects of symbiotic fixation would be facilitated. These are also the reasons for the corresponding tests on non-legume nodules.

The results now reported for such nodules confirm that the organs are nitrogen-fixing. Detached nodules of *Hippophaë*, *Casuarina*, and *Alnus* exposed to excess free ^{15}N achieve a final enrichment which, though averaged out in the present data over the whole nodule nitrogen, is quite substantial, ranging up to the 0·645 atom per cent. gain shown by *Hippophaë* nodules. Tove, Niss, and Wilson (1950) were unable to demonstrate consistently the occurrence of fixation in detached legume nodules when the whole of the nodule nitrogen was assayed, the overall enrichment being often insignificant, as was also found by Bond (1955) with Soya bean nodules. In subsequent Madison work analyses have been confined to the acid-soluble, metabolically active fraction of the nodule nitrogen in order to secure more readily measurable enrichment. One of the highest enrichments reported on this basis is 0·482 atom per cent. for a particular sample of Soya bean nodules exposed to 31 atom per cent. excess ^{15}N (Magee and Burris, 1954). Considering that the fraction of nitrogen assayed represented only a small (though not actually stated) part of the total nodule nitrogen, it seems certain that even in these particularly active nodules the overall enrichment would have been small. If the fixation is considered with reference to the dry weight of the nodules rather than to their total nitrogen content, then the legume nodules show up slightly more favourably, since weight for weight they contain more nitrogen. Thus values for the percentage nitrogen content of nodule dry matter obtained in Glasgow experiments are as follows: Soya bean 4·5, pea 5·9, *Alnus* 3·6, *Hippophaë* 3·4, *Myrica* 3·4, *Casuarina* 3·0.

The greater final fixation achieved by the nodules of *Hippophaë*, *Alnus*, and *Casuarina* is obviously due largely to the longer persistence of fixation after detachment. Aprison and Burris (1952) found that fixation diminished rapidly in Soya bean nodules after detachment, being practically stopped after 6 hours. Fig. 1 shows that fixation continued for some hours longer in *Hippophaë* and *Alnus*, and that in the former there was probably still appreciable fixation after 24 hours. As noted by Aprison and Burris for legumes, the fact that in the non-legumes the rate of fixation did diminish with time is evidence that the fixation was due to nodule action and not to the development of contaminating nitrogen-fixing organisms under the non-aseptic conditions of the experiments. One of various possible explanations of the eventual falling-off of fixation is that metabolic materials required in fixation become exhausted. According to Burris (1956) in unpublished work Magee has found that fixation in sliced legume nodules can be materially increased (and possibly prolonged) by supplying sucrose. Perhaps the non-legume nodules are better provided with reserves.

Owing to lack of comparable data it cannot be stated whether the detached non-legume nodules show more rapid fixation than those of the legume type when fixation in both is most active, i.e. immediately after detachment. This is a point of considerable interest from the standpoint of the suitability of the material to metabolic studies. In the work now reported it was preferred that all the nodule samples in a particular experiment should be gassed simultaneously, with the result that an appreciable interval elapsed between the commencement of nodule detachment and exposure to ^{15}N, as indicated in the footnotes to the Tables. In recent Madison legume experiments this delay has been reduced to a few minutes, and by proceeding similarly with the non-legumes still higher rates of fixation than those now reported may be obtainable.

It is of interest to inquire how the fixatory activity of detached nodules compares with that of normal, attached, nodules. Unfortunately calculations of nodule activity cannot be made from isotopic tests with whole plants, such as those in Table I, because of the rather rapid leakage of ^{15}N from the vicinity of the roots, through the shoot tissues (Bond, 1955), but calculations from other data indicate that the nodules of young *Alnus* plants, when at the height of their activity, must fix something of the order of 50 per cent. of their nitrogen content per day, while in *Hippophaë* the figure is probably nearer to 100 per cent. The tests with detached nodules were made at the season of maximum nodule activity, and calculations from the data indicate that the fixation of ^{14}N and ^{15}N finally achieved was as follows, assuming that both forms of nitrogen are fixed equally readily, as demonstrated by Burris and Miller (1941) for other material:

Hippophaë (Fig. 1), 1·8 per cent. of the total nodule nitrogen;
Alnus (Fig. 1), 0·5 ” ” ” ” ;
Casuarina (Table II), 0·9 ” ” ” ” .

Obviously the fixation achieved in the 24 hours following detachment is only a small fraction of that of normal nodules. Only for a very short time after detachment can the rate of fixation possibly approach the normal value.

The result of the experiment on fixation by detached *Myrica* nodules at different oxygen tensions shows some agreement with that of a previous one with whole plants of *Myrica* in which the root systems were exposed to different oxygen levels during development (Bond, 1952). There also the most clearly marked effects were shown when the oxygen level was maintained below 5 per cent. The result with the detached *Myrica* nodules differs from that of an earlier experiment of the same type with *Alnus* nodules (Bond and MacConnell, 1955), where a considerably greater sensitiveness to the lowering of the oxygen supply from the normal level seemed to be indicated. Generic differences in the oxygen response may exist, and the fact that the *Alnus* nodules were more active in fixation may also be significant. It was, however, pointed out that the *Alnus* experiment was open to the criticism that the ratio of volume of gas to weight of nodules was rather low, so that marked reduction in oxygen tension was likely to have occurred during the period of incubation. This and other aspects of the fixation shown by non-legume nodules are being investigated further.

ACKNOWLEDGEMENTS

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The Development and Behaviour of Mycelial Strands in *Merulius lacrymans* (Wulf.) Fr.

I. Strand Development during Growth from a Food-base through a Non-nutrient Medium

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ABSTRACT

Strand development by *Merulius lacrymans* was followed by direct observation of the surface growth of the fungus from a wood food-base over a moist non-nutrient medium contained in glass tubes. The first-formed mycelium contained no visible strand-like aggregations and strand initials became apparent in a relatively narrow physiological age of mycelium behind the mycelial margin. New growth in the older parts of the mycelium consisted of increase in thickness of some of these strand initials, except where 'sprouting' of strands occurred. Visible surface maturation of strands did not take place and some strands continued to increase in thickness over the longest experimental period used. In tubes the rate of strand formation remained constant with increasing distance from the food-base and longitudinal systems of strands were built up. Evidence is given that altering the size or distance away of the food-base affects the density of mycelium and strands more than it affects the growth-rate of individual hyphae of the mycelial margin.

I. INTRODUCTION

THE terms 'mycelial strand' and 'rhizomorph' have both been used in the literature to describe the linear aggregations of vegetative hyphae which occur in many fungi, especially basidiomycetes, e.g. Bommer (1896), Townsend (1954). Although the mature structures may be similar, Garrett (1956*a*) has distinguished between these two types of aggregation on developmental grounds. Thus a simple mycelial strand consists of a loose federation of hyphae, increasing in thickness by the accretion of hyphae from the base (e.g. *Helicobasidium purpureum*) whereas a typical rhizomorph, e.g. that of *Armillaria mellea*, is a fully autonomous apically growing organ. Information on the development of these structures is at present limited to observations of only a few fungi and Garrett recognizes that intermediates between these defined types probably exist. Moreover little is known of the developmental relation of strands and rhizomorphs with ordinary vegetative mycelium. Garrett (1953) has shown that in *A. mellea* the range of nutritional conditions favouring rhizomorph initiation and growth are both narrower than those permitting mycelial growth. This observation is comparable with the general conclusion of Hawker (1950) that the range of environmental conditions permitting sporulation is narrower than that permitting mycelial growth.

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Both strands and rhizomorphs occur in nature either in connexion with fruit bodies or extending from a food-base through non-nutrient media to a new food-supply. Although it is clear that nutrients and water are conducted along such aggregations, little experimental evidence on the relative efficiency of conduction in mycelium and mycelial aggregations is available. Schütte (1956), who summarizes earlier investigations of fungal translocation, describes rapid active polar transport of fluorescein in definite zones of young agaric stipes. He could not distinguish between translocating and non-translocating hyphae on anatomical grounds. Garrett (1956*a*) has approached the problem of the biological advantage of such structures from a different angle. He noted that organs of mycelial migration are typically produced not only by many root disease fungi, especially of woody plants, but also by saprophytic lignin and cellulose decomposing fungi. He had demonstrated that for successful infection of the host roots an adequate inoculum potential of the parasitic fungus was required to overcome host resistance. In *Armillaria mellea* this inoculum potential is supplied by the rhizomorphs. By analogy with the behaviour of parasitic fungi, Garrett postulated that a similar inoculum potential was needed for successful colonization of a lignin substrate by a saprophytic fungus and that this inoculum potential was supplied by mycelial aggregations. In experiments with the cultivated mushroom Garrett provided some evidence in support of this hypothesis.

Merulius lacrymans (Wulf.) Fr. has long been recognized as the principal cause of 'dry rot' of building timber in Europe. One of the most striking features of recorded outbreaks of dry rot is the frequent occurrence of 'mycelial strands' spreading from a wood food-base for considerable distances over and through non-nutrient material. These strands may reach 5–8 mm. in diameter (Cartwright and Findlay, 1946) and Butler (1943) recorded their spread for 10 ft. along a steel joist and for 15 ft. from the nearest timber through a brick wall. Moreover the strands contain differentiated hyphae, including very wide 'vessel hyphae' (Falck, 1912). These observations suggested that *M. lacrymans* would provide good material for studying the problems of development and behaviour of linear mycelial aggregations in a saprophytic fungus.

As early as 1885 Hartig stated that *M. lacrymans* spread as delicate scarcely visible mycelium in which quite fine, abundant ramifying strands developed. These strands later often increased in diameter to the thickness of a pencil. Falck (1912), in a detailed monograph on the fungus, described a number of experiments in which he considered strand configuration. He concluded that spatial contraction of the original mycelium, supplementary new building, and the flowing together of the contents of the individual hyphae are all essential parts of strand development. He contrasted this behaviour with that of true rhizomorphs which have a fixed origin and centrifugal apical growth, and also that of sclerotia in which the structure is delimited on all sides. Since 1912 much literature on the fungus has accumulated (Cartwright and Findlay, 1946) but little experimental work on strand development has been reported.

In this first paper experiments designed to clarify the developmental relation of mycelium and strands under a single simple system of growth are described.

II. MATERIALS AND METHODS

All experiments were carried out with a single isolate of *M. lacrymans* supplied by the Forest Products Research Laboratory, Princes Risborough. No attempt was made to ensure the genetical uniformity of the material and mycelial inoculum was used throughout. The stock culture was maintained on slopes of 2·5 per cent. malt agar kept under paraffin oil. The isolate continued to form good strands throughout the period of investigation and no changes in its behaviour were evident.

Strand development by *M. lacrymans* was followed by direct observation of the surface growth from a food-base over a moist non-nutrient medium contained in glass tubes. A similar technique has been used by various investigators (King, Loomis, and Hope, 1931; Blair, 1943; Garrett, 1954 and 1956*b*).

For most of the experiments fragments of porous plant pot sieved to particle size 3–6 mm. were used as the non-nutrient medium. The chippings were washed by soaking in concentrated hydrochloric acid and then rinsing in a continuous stream of tap water for several hours before drying in an oven. The moisture-holding capacity of the chippings was determined using the perforated box method of Keen and Raczkowski (1921) and moisture contents were expressed as a percentage of the moisture-holding capacity (m.h.c.). Glass tubes of internal diameter 1·9 cm. and length 25 or 38 cm. were packed with dry pot chippings, the required amount of water was added to each tube, and the tubes were left overnight. The column of filling in each tube was supported at both ends by nylon plugs placed within the ends of the tubes, since *M. lacrymans* utilized cellulose cotton wool. In some experiments tubes were sterilized by wrapping in grease-proof paper and autoclaving for 30 mins. at 15 lb. pressure. The tubes were weighed before and after autoclaving and any that changed in weight by more than 1 g. were rejected.

Although good growth occurred from both sawdust and wood inocula under sterile and unsterile conditions, growth from agar inoculum discs did not take place where conditions were unsterile and was poor and transitory under sterile conditions. Wood inoculum was used as a food-base in most experiments. Blocks of *Pinus sylvestris* sapwood 2·5×1·2×1·2 cm. were dry sterilized in an oven for 18 hours at 100° C. (Cartwright and Findlay, 1946) and colonized with *M. lacrymans* by placing them on the surface of actively growing cultures of the fungus on 2·5 per cent. malt agar in screw-cap jars. The age of colonization of the inoculum blocks varied between but not within experiments (from 9 to 18 weeks after inoculation). Where experiments were carried out under unsterile conditions, the inoculum blocks were first cleaned of surface mycelium and then incubated for 3 days in a moist chamber; any that did not develop a covering of mycelium of *M. lacrymans* were rejected. The inoculum was usually placed at one end of the column of filling adjacent to the nylon plug. The ends of the tubes were capped with moisture-proof 'Cellophane'. Since the optimum temperature for growth of this isolate of *M. lacrymans* was found to be close to 22·5° C., tubes were incubated either at room temperature (14–21° C.) or at 22·5° C.

In order to compare strand development in both space and time, some numerical measure of the degree of stranding in tubes was essential and a sampling technique was devised. Using a microscope with a 2-in. objective and reflected light, the number and diameter of all strands 15 μ or more in diameter which crossed a marked ring or rings around each tube were recorded.

Since major stranding extended along the length of the tubes a maximum number of strands cut each 'ring transect' at right angles. The difficulty of defining the lower limit of diameter of a strand could only be avoided by including only those strands thicker than a certain arbitrarily chosen diameter, 15 μ (0·5 division of the eyepiece micrometer scale used for this work). Strand diameter was recorded to the nearest 0·5 scale division ($\pm 7{\cdot}5\,\mu$). The use of such ring transects enabled observation of the *same* mycelium to be made at different times, so that one source of variation was eliminated.

Details of this tube technique are shown diagrammatically in Fig. 1. In spite of all precautions, considerable variation in mycelial growth occurred between replicate tubes. It is probable that this was due principally to differences in the inoculum and to unequal growth through the centre and along the glass walls of the tubes.

III. CHANGES IN MACROSCOPICALLY VISIBLE STRAND DEVELOPMENT WITH TIME

Preliminary Experiments

An experiment was set up to investigate the effect of different particle sizes of various non-nutrient materials on the development of mycelium and strands from an inoculum food-base. Washed fragments of plant pot, gravel, flint chippings, limestone chippings, and pumice were all sieved into different size fractions and the moisture-holding capacity of each fraction was determined. Three unsterile replicate tubes of each series were prepared, all at 50 per cent. m.h.c., and each tube was inoculated at one end with a wood block colonized by *M. lacrymans* and incubated at room temperature.

The results (Table I) show that on all media except pumice growth was slow and irregular where the particle size was small, and that good growth occurred on all media at the larger particle sizes. It is possible that the adverse effect of small particle size was associated with poor aeration, similar to that observed by Blair (1943) for *Rhizoctonia solani*. Since porous pot chippings of particle size 3–6 mm. had a relatively high moisture-holding capacity (40 g. per 100 g.), and since mycelial growth was good and easy to see against the red colour of the pot, this substance was selected for use in further experiments. During this experiment it was observed that *M. lacrymans* grew out from the food-base, first appearing macroscopically as undifferentiated mycelium which continued to grow to the end of the tube. For convenience this first colonizing mycelium is termed 'original'. Strands first became macroscopically visible at some distance behind the mycelial margin and most of them extended along the length of the tubes (Fig. 1*a*, *b*).

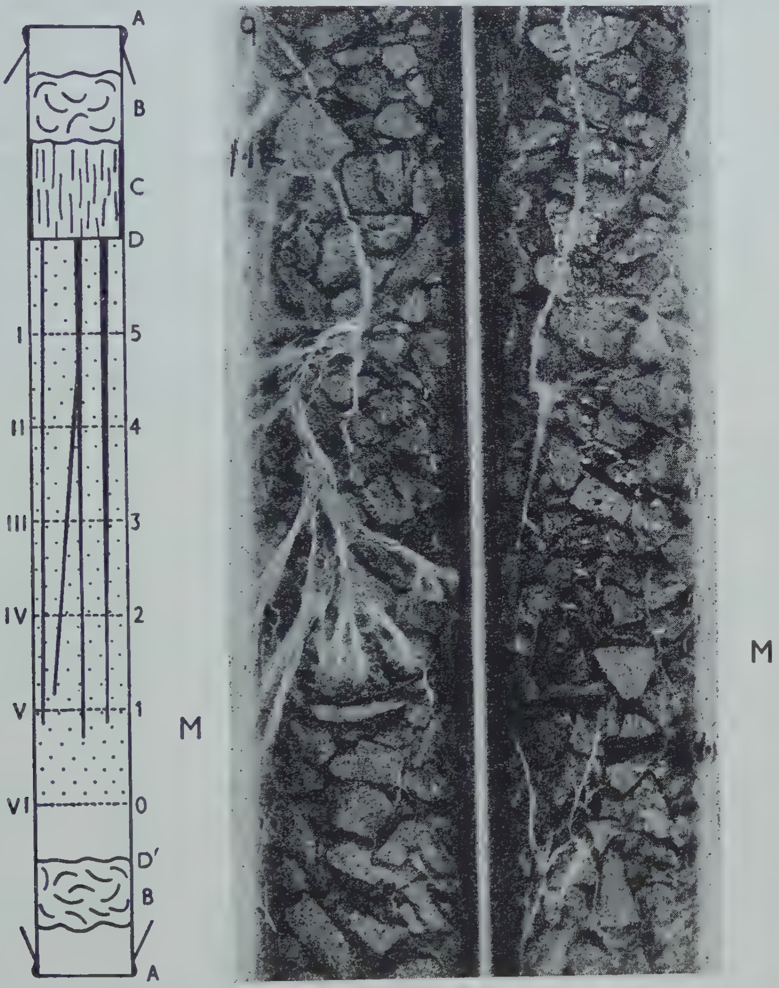

FIG. 1 (*a*). FIG. 1 (*b*).

FIG. 1 (*a*). Diagram of the tube technique, showing mycelial development by *M. lacrymans* 6 weeks after inoculation. A, 'Cellophane' cap; B, nylon wool plug; C, wood inoculum; D–D', column of moist porous pot chippings; I–VI, positions of mycelial margin at successive weekly intervals after inoculation; 0–5, age of original mycelium at successive positions in the tube. FIG. 1 (*b*). M. The origin of sprout mycelium from strands of *M. lacrymans* in tubes containing moist porous pot chippings. Left: extensive fan-shaped mycelium with strands has arisen from one part of the original strand. Right: sparse sprout mycelium has arisen from several points along a strand (upper half of photograph).

(Magnification × $1\frac{1}{2}$.)

The moisture content of porous pot most suitable for prolonged observations of strand development was also investigated. Test-tubes containing sterile porous pot at 20, 40, 60, and 80 per cent. m.h.c. were inoculated with colonized sawdust and after capping with moisture-proof 'Cellophane' were kept in a polythene bag at 22·5° C.; no further water was added. Linear extension of the mycelium was most rapid at the lowest moisture content, 20 per cent. m.h.c. (40 mm. per week compared with 30, 23, and 25 mm. per week at 40, 60, and 80 per cent. m.h.c. respectively). Nevertheless strand development was poor at an initial moisture content of 20 per cent. m.h.c. and mycelial growth and stranding at 60 and 80 per cent. m.h.c. were irregular and depressed compared with those at 40 per cent. m.h.c. Further experiments were therefore carried out at an initial moisture content of 40–50 per cent. m.h.c.

TABLE I

Mycelial Growth and Strand Development by M. lacrymans *in various Non-nutrient Media, Graded for Different Particle Sizes*

Material and size of particle (mm.)	pH	m.h.c. (g. per 100 g.)	Mean mycelial growth-rate (mm. per wk.)	Mycelial margin	Stranding	Visibility
Pot >6	6·0	35	35	Even, thin	Numerous	Good
3–6		40	33	,,	,,	
1·5–3		51	25	Irregular	Few	
0·5–1·5		52	16	Irregular	Difficult to define	
Gravel >6	6·8	18	29	Even, thin	Numerous	Moderate
3–6		23	29	,,	,,	
1·5–3		21	22	Even	Reticulum	
0·5–1·5		23	13	Irregular	Difficult to define	
Flint 3–6	7·0	17	25	Irregular	Moderate	Good
1·5–3		26	21	,,	,,	
0·5–1·5		30	15	Very sparse	Poor	
Limestone	9·2	21	26	Even	Few, thick, yellow	Moderate
Pumice 0·5–1·5	6·6	95	26	Dense, brown	Dense	Poor

Changes in Stranding with Time, at a Constant Distance from the Food-base

An experiment was set up in order to define the relation between strand development and age of the original mycelium. During the previous experiments loss in weight of the tubes occurred with time. This loss may have been due both to evaporation of water from the tubes and to loss of volatile metabolic products of the fungus. Since it was thought that a drift in water content might have some effect on the course of strand development, in this experiment two parallel series of sterile tubes were set up containing chippings at 40 per cent. m.h.c. The tubes were weighed to 0·1 g., the control series were left as before and the tubes of the second series were maintained at their original weights by fortnightly additions of sterile water at the end farthest from the inoculum. Since no allowance was made for losses in weight due to metabolic activity of the fungus, the moisture level in the second series was maintained at or above the initial level. The position of the mycelial margin was recorded weekly and strand assessments were made 7·5 cm. from the food-base at weekly intervals after the mycelial margin had reached this position.

During the experimental period the control series lost weight regularly (0·23 g. per week) and the mean weekly loss in the maintained series was slightly higher, 0·28 g., probably due to loss at uncovering for aseptic addition of water. Mean advance of the mycelial margin during the 26 days from 6 to 32 days after inoculation was the same in both series, 9·4 cm., and strand development followed the same general course under these sterile conditions as was observed earlier under unsterile conditions. Strand assessment results (Table II) show that in both series the number of strands reaching the lower diameter limit was high during the first 2–3 weeks after development of the original mycelium, but that thereafter changes in strand number were very small. Since the same strands were present at the different times, this observation indicated that strand initiation occurred only relatively early behind the mycelial margin. On the other hand, increase in thickness, at least of the widest strand in each tube, continued over the whole period of observation.

TABLE II

Strand Development by M. lacrymans *7·5 cm. from the Food-base in Tubes Containing Porous Pot*

(a) Changes with Time in Mean Strand Number (to the nearest whole number)

Treatment	Strand diameter groups (scale divisions*)	Age of original mycelium (weeks)							
		1	2	3	4	5	6	7	8
Water added periodically to maintain the moisture content	0·5–1	0·3	9	8	7	6	5	6	—
	1·5–3	0	2	4	6	5	6	5	—
	3·5–7	0	0	0	0·3	1	1	2	—
	7·5–15	0	0	0	0	0	0	0·1	—
	All strands	0·3	10	12	13	13	12	13	—
Moisture content not maintained	0·5–1	0	9	12	10	9	8	5	7
	1·5–3	0	1	4	6	6	7	8	6
	3·5–7	0	0	0·3	1	3	2	2	2
	7·5–15	0	0	0	0	0	0·3	1	1
	All strands	0	10	17	17	18	17	16	16

(b) Changes with Time in Mean Diameter of the Thickest Strand per Transect (in scale divisions)*

Treatment	Age of original mycelium (weeks)							
	1	2	3	4	5	6	7	8
Water added periodically to maintain the moisture content	0	1·5	2·1	2·6	3·1	3·9	5·0	5·4
Moisture content not maintained . .	0	1·4	2·4	3·4	4·4	5·9	6·0	6·9

* 1 scale division = 30 μ.

From the changes in diameter distribution with time (Table II), it can be seen that although some strands continued to increase in thickness over the whole time, the rate of increase was slower in many and seemed to cease altogether in some. Thus, of the strands which were placed in the 0·5–1 diameter group 3 weeks after formation of the original mycelium, over half remained in the same diameter group 4 and 5 weeks later. The method of observation employed was not capable of discriminating between living mycelium full of contents and that which was empty, but it is possible that some or all of the non-progressive strands lost their contents. Further evidence of this pattern of development is provided by a series of detailed transects along the same line round a single tube at different times (Table III).

TABLE III

Changes in Diameter (in scale divisions) of Individual Strands of* M. lacrymans *as shown by Successive Transects along the same Line round a Single Tube*

	Age of original mycelium (weeks)					
Strand	1	2	3	4	6	8
A	<0·5	1·5	2·5	5·5	6·5	9
B	<0·5	0·5	3	4	6	6
C	<0·5	0·5	1	1	1	1
D	<0·5	0·5	4	4·5	4·5	4·5
E	<0·5	1·5	4	6	6·5	9
F	<0·5	0·5	1	1	1	1
G	<0·5	1	0·5	0·5	0·5	0·5
H	<0·5	1	1	1	1	1
I	<0·5	0·5	0·5	<0·5	0·5	0·5
J	<0·5	<0·5	<0·5	<0·5	0·5	0·5
Total no. strands (>0·5 s.d.*)	0	9	9	8	10	10

* 1 scale division (s.d.) = 30μ.

Of the nine strands which had been initiated by the end of the second week after formation of the original mycelium, only four increased appreciably in diameter over the following 6 weeks. Some form of strand preference had occurred whereby certain strands developed at the expense of others and longitudinal systems of strands were built up, which might branch or reunite along the length of the tubes.

A comparison of the two series (with and without periodic addition of water) shows that the general pattern of development was the same in both, and any difference between the two was related to overall amount of strand development. The variation between replicates was too large for the significance of this difference to be established. With an initial moisture content of 40 per cent. m.h.c. development in porous pot continued over 14 weeks without addition of water. For subsequent experiments water-content adjustments were made infrequently, usually every 4 weeks.

Effect of Distance from the Food-base

During earlier experiments it was observed that the growth-rate of the mycelial margin changed little with increasing distance from the food-base (Fig. 1*a*, *b*). A single unsterile tube 60 cm. long was set up and inoculated at one end with a block of colonized wood, 2·5 cm. long. The tube was kept at room temperature and the moisture content was maintained at 40 per cent. m.h.c. Over 18 weeks, during which the mycelium grew a total of 41·9 cm., the extension of the mycelium in successive weeks was as follows: 21, 20, 15, 20, 22, 20, 24, 27, 24, 24, 27, 23, 22, 23, 24, 8, 0, 0 mm. Over 15 weeks no diminution in growth-rate occurred with increasing distance from the food-base and subsequently growth ceased abruptly.

The course of strand development was followed at different distances from the food-base in a further experiment. Strand assessments were made at positions 5, 10, 15, and 20 cm. from the food-base at weekly intervals after the formation of the original mycelium at each position, in each of five replicate sterile tubes. The moisture content of the porous pot was 40 per cent. m.h.c. and the tubes were incubated at 22·5° C. Results are shown in Table IV.

TABLE IV

Strand Development by M. lacrymans *at Different Distances from the Food-base*

(*a*) *Changes in Mean Strand Number with Time* (*to the nearest whole number*)

Distance from food-base (cm.)	Growth-rate (mm. per week)	Age of original mycelium (weeks)					
		1	2	3	4	5	6
5	—	2	14	21	20	19	15
10	32	2	12	12	9	10	10
15	38	3	10	9	8	7	7
20	35	0	6	9	9	—	—

(*b*) *Changes in Mean Strand Diameter with Time* (*in scale divisions**)

Distance from food-base (cm.)	Age of original mycelium (weeks)					
	1	2	3	4	5	6
5	0·5	0·7	1·2	1·5	1·5	1·7
10	0·5	0·9	1·1	1·4	1·4	1·6
15	0·7	0·9	1·0	0·9	1·0	1·0
20	0	1·0	1·2	1·2	—	—

* 1 scale division = 30 μ.

The growth-rate of the mycelial margin changed little and the rate of development of strand initials behind the margin remained constant, within the limits of sensitivity set by observations at weekly intervals, with increasing distance from the food-base. There was a consistent reduction in mean number of strands with increasing distance from the food-base, except at 20 cm. where the amount of stranding may have been biased by close proximity to the end of the porous pot column. Since mean strand diameter also tended to decrease, the changes reflected a reduction in the amount of mycelium involved in strand development and could not be ascribed to branching and reuniting of strands along the length of the tubes. It was not clear how far these changes were due to a drift in environmental conditions with time and how far they were due to a reduction in nutrient-supply as the mycelial system expanded.

Further experiments in which the effect of preventing apical growth of the mycelium was investigated failed to provide any evidence that a mechanical block altered the rate or increased the amount of stranding.

IV. EFFECT OF POSITION AND SIZE OF THE FOOD-BASE

Effects of Disconnecting Food-base, and of Providing a Subsidiary

An experiment was set up to investigate the part played by the food-base in strand development. Four experimental series were set up:

1. Control, inoculum intact, no new food-supply.
2. Inoculum removed after mycelial growth for 12·5 cm., no new food-supply.
3. Inoculum intact, a new food-supply at 12·5 cm.
4. Inoculum removed after growth into the new food-supply at 12·5 cm.

The new food-supply consisted of 1 g. coarse (*Pinus spp.*) sawdust per tube and it was arranged so that the centre of each sawdust column was 12·5 cm. from the inoculum block. Each column of porous pot was adjusted to 40 per cent. m.h.c., and a further 1·8 ml. water was added to each tube containing sawdust. The five replicate tubes of each treatment were sterilized and, after inoculation, were incubated at 22·5° C. The position of the mycelial margin was recorded weekly and strand assessments were made at positions 5 and 10 cm. from the inoculum blocks at weekly intervals after the passage of the mycelial margin. The results are shown in Table V.

It was observed that the growth-rate of the mycelial margin was reduced as it approached and grew through the sawdust. Where no new food-supply was present, the growth-rate of the mycelial margin fell abruptly and ceased almost entirely within the first week after removing the food-base. Similarly all further development of strands ceased and the mycelium became brown and appeared moribund. This result, which was confirmed by another similar experiment, suggested not only that materials available from the pot chippings were inadequate for growth of *M. lacrymans*, but also that supplies contained in the mycelium as it advanced were insufficient for further development of strands. These experiments did not establish whether the fungus was dependent on the food-base for water as well as for nutrients.

After the food-base had been removed from the tubes in which colonization of sawdust had begun, the original mycelium persisted, the principal strands continuing to appear white and healthy. Although no backward growth of undifferentiated mycelium from the colonized sawdust occurred, ultimately some extension of mycelium into the nylon plug at the inoculum end took place in all five replicates. Strand assessments showed that not only did the strands persist but also that many of them increased in diameter. A comparison of the various series suggested that some disturbance to the course of

TABLE V

Effect of Altering the Food-base on Strand Development by M. lacrymans

(*a*) *Mean Weekly Growth-rate* (*mm.*)

	Time in weeks (from inoculation)						
Treatment	4	5	6	7	8	9	10
1. Control, inoculum intact	31	32	39	31	Overgrown		
2. Inoculum removed	28	29	‖ 5	0	0	—	—
3. New food-base, inoculum intact	24	23	16	23	16	24	36
4. New food-base, inoculum removed	26	18	21	‖ 16	20	27	32

(*b*) *Changes in Mean Strand Number with Time*

		5 cm. from inoculum					10 cm. from inoculum				
Age of original mycelium (weeks)		1	2	3	4	5	1	2	3	4	5
Treatments:	1 (as above)	2	14	21	20	19	2	12	12	9	10
	2	3	14	‖ 15	12	10	‖ 0	0	0	0	0
	3	1	15	24	20	15	0	4	4	4	6
	4	0	14	24	‖ 19	15	0	‖ 5	6	5	5

(*c*) *Changes in Mean Diameter of the Thickest Strand per Transect* (1 *scale division* = 30μ)

		5 cm. from inoculum					10 cm. from inoculum				
Age of original mycelium (weeks)		1	2	3	4	5	1	2	3	4	5
Treatments:	1 (as above)	0·8	1·1	2·5	3·7	3·9	0·5	1·5	2·4	3·2	3·3
	2	0·5	1·3	‖ 1·5	1·3	1·3	‖ 0	0	0	0	0
	3	0·5	1·7	2·9	3·2	3·4	0·5	0·9	1·4	1·5	1·6
	4	0·5	1·2	2·8	‖ 3·7	4·3	0·5	‖ 0·9	1·3	2·1	2·5

‖ indicates time of removal of inoculum.

strand development occurred after colonization of the new food-supply, both where the inoculum remained intact and where it was removed. Nevertheless it may be concluded that where effective connexion with the new food-supply had occurred, strands continued to increase in diameter in spite of reversal in the direction of nutrient-supply.

Effect of Volume of Food-base

Colonized blocks of uniform cross-section (standard 1·2×1·2 cm.) and different lengths, 2·5, 1·25, and 0·75 cm., were placed at the end of unsterile 25 cm. tubes with the filling at 40 per cent. m.h.c. The five replicate tubes of each series were kept at room temperature and a single assessment of stranding was made at two positions on each tube 14 weeks after inoculation (Table VI).

Although growth-rates were not recorded at regular weekly intervals, it was observed that after an initial lag period, which was longer for the smallest blocks, the growth-rates of all three series were closely parallel. A general reduction in the amount of mycelium taking part in strand development (i.e. in both mean strand number and mean strand diameter) occurred with reduction in size of the food-base. Although no account was taken of the relative ages of the original mycelium at assessment in the different tubes, this factor

TABLE VI

Growth and Stranding by M. lacrymans *from Food-bases of Different Volumes*

	Mean extension of mycelial margin (mm.)		Strand development 14 weeks after inoculation			
			Mean no. strands		Mean diameter strands (s.d.)†	
Length of food-base	0–18 days	18–36 days	5 cm.*	10 cm.*	5 cm.*	10 cm.*
2·5 cm.	54	89	6·4	7·2	2·9	2·7
1·25 cm.	50	90	4·8	5·8	3·1	2·8
0·75 cm.	25	81	4·0	4·4	1·7	2·1

* Indicates distance from food-base.

† 1 s.d. (scale division) = 30 μ.

was unlikely to have affected the results greatly since differences in age of the original mycelium were of the order of 1–2 weeks out of the total growth period of 14 weeks.

These observations suggested that the whole resources of the food-base were exploited during mycelial development; differences in rate of supply of nutrients caused by variation in size of the food-base were reflected in reduced lateral development of mycelium rather than in reduced growth-rate of individual hyphae of the mycelial margin.

V. SPROUTING

The term 'sprouting' is used to describe the development of free hyphae from the strand-mycelium complex behind the mycelial margin. The new hyphae grew out as progressive fan-shaped mycelium in which strands subsequently developed and this was the only type of growth behind the margin which did not consist of increase in thickness of strands. In some instances sprouts could be seen arising from strands (Fig. 1*b*) but in others their origin was hidden amongst the pot chippings.

Sprouting occurred late in development in some tubes, after growth of *M. lacrymans* to the end of the pot column. It was not associated with density or paucity of the preformed mycelium, nor with exhaustion of the food-base. Since it occurred most frequently in experiments which were carried out in a 22·5° C. constant temperature room containing a fan and in tubes to which no water was added after setting up, it seemed likely that sprouting was associated with drying out of tubes. Five series of 25 cm. sterile tubes were therefore prepared as follows:

1. Water level maintained at 40 per cent. m.h.c. by fortnightly additions of sterile water.
2. Water content maintained at 60 per cent. m.h.c.
3. Originally at 40 per cent. m.h.c. but no subsequent additions of water.
4. Maintained at 40 per cent. m.h.c. until the mycelial margin reached 12·5 cm. from the food-base; then 3·6 ml. water added to bring to 60 per cent. m.h.c., at which subsequently maintained.
5. Originally at 40 per cent. m.h.c., with a single addition of 3·6 ml. water when the original mycelium at 7·5 cm. from the food-base was 6 weeks old.

The tubes were incubated at 22·5° C. and records of the occurrence of sprouting were made (Table VII).

TABLE VII

Number of Tubes in which Sprouting by M. lacrymans *occurred during Successive Weeks after Inoculation*

Treatment (see text)	Weeks from inoculation						Not observed up to 15th week	Total no. tubes
	9	10	11	12	13	14		
1. 40% maintained	0	0	0	4	1	0	3	8
2. 60% maintained	0	0	0	0	0	0	6	6
3. 40% not maintained	0	3	2	2	0	0	0	7
4. 40%→60%	0	0	0	0	0	0	6	6
5. 40%+3·6 ml. late	0	1	5	0	0	0	1	7

The regularity in time of occurrence of sprouting suggested that it was not due to chance disruption of the strand mycelium. Sprouting was most abundant in those tubes to which no water was added after inoculation (Series 3); it did not occur where the moisture content was maintained at 60 per cent. m.h.c. nor where it was brought to 60 per cent. m.h.c. before mycelial growth to the end of the tubes (Series 2 and 4 respectively). Moreover, development of already initiated sprout mycelium continued after addition of water which more than restored the original moisture content (Series 5). Sprouting was not simply due to drying out to a certain level, since it occurred, although late, in some of the tubes maintained at 40 per cent. m.h.c. by fortnightly additions of water.

VI. DISCUSSION AND CONCLUSIONS

Observations of visible mycelial growth over the surface of porous pot contained in tubes have helped to elucidate the mechanics of strand development at a macroscopic level in *M. lacrymans*. During growth from a localized food-base into this non-nutrient medium, the leading hyphae of the mycelial margin showed no tendency to grow in tufts or other strand-like associations. Although limiting the number and size of available spaces for growth of hyphae may favour fortuitous aggregation, this limitation is not necessary for strand development; strands developed freely in moist air away from any surface. Strand development proceeded behind the mycelial margin and strand initials were formed only in a relatively narrow physiological age of mycelium. Further growth behind the margin, which depended on connexion with a food-base, consisted almost entirely of increase in thickness of some of the strand initials, resulting in the production of more or less longitudinally arranged strands. The time sequence of strand development was not appreciably altered either at different distances from the food-base or where apical growth of the mycelium was checked by a mechanical barrier. Moreover, development continued where the direction of nutrient-supply was reversed. There was no visible evidence of a surface maturation of the strands, e.g. formation of a delimited cortex as has been described for *Phymatotrichum* *omnivorum* (Rogers and Watkins, 1938) and for various other fungi (Townsend, 1954). It would appear that strand thickness was limited only by the time for development and the nutrients available. Nevertheless it was clear that more strand initials were formed than subsequently developed into strands. Theoretically growth from an inoculum of *M. lacrymans* would tend to form a sphere of radiating hyphae and therefore some constraint on the growth and branching of the leading hyphae occurred in the tubes. Branching of strands to form a more or less dendroid network could be seen. Further growth of free hyphae behind the mycelial margin was only observed where sprouting took place. Strand initials were formed in this sprout mycelium in a similar way to those in the original mycelium. The conditions favouring sprouting have not been established, but it seems likely that one of the important factors is moisture status.

These observations of the time sequence of strand development substantially confirm the work of Hartig (1885) and Falck (1912), but they indicate that the spatial contraction of the original mycelium described by Falck plays at most only a small part in strand development. There is good evidence that strands are formed by growth of new hyphae. The tapering form of strands from the food-base does not necessarily imply that all growth of new hyphae is directly from the food-base. A possible explanation of strand preference is that a chance initial advantage in nutrient-supply is magnified as growth proceeds. Since strand initials are first apparent close to the mycelial margin further clarification of these points may be obtained by observations of the behaviour of individual hyphae close to the mycelial margin. Such observations will be described in a subsequent paper.

On developmental grounds the 'strands' of *M. lacrymans* may be contrasted with the apically growing rhizomorphs of *Armillaria mellea* (de Bary, 1887). Moreover, the uniform macroscopic appearance of the mycelium of *M. lacrymans* growing in a moist chamber from a food-base may be contrasted with the tufted mycelial habit of some other saprophytic fungi, e.g. *Phallus impudicus*, under similar conditions. In the latter loosely woven apically growing units are apparent even in the first-formed mycelium. Thus, although the mature structures of *M. lacrymans* are highly specialized, they may be regarded as typical mycelial strands as defined by Garrett (1956*a*).

The experiments suggest that variations in size and distance away of the food-base affected the amount of lateral development of mycelium and strands more than it affected the growth-rate of individual hyphae of the mycelial margin. No indication was found that moisture content of the medium affected the proportion of strands to undifferentiated mycelium, but the effect of relative humidity was not investigated.

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The Growth of Scions under Conditions of Extreme Vigour

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With Plates XX and XXI and four Figures in the Text

ABSTRACT

In order to ascertain growth-rates of scions free from rootstock limitations, shoot- and root-scions were induced to grow very rapidly by grafting them on to heavily pruned rootstocks. Under these conditions the growth-rates of shoot-scions did not follow the same order as the root-scions of the same varieties; the growth-rates of root and shoot scions relative to one another were in accordance with the hypothesis that the growth-rate of a composite tree was the resultant of the growth-rates of its component varieties. *Varietal* differences in shoot weight were attributable largely to differences in the proportion of leafage on the shoots, average increments in shoot weight per gram of leaf being nearly the same for all varieties; *within* varieties, the lighter shoots had less increment per gram of leaf than the heavier, despite, in some cases, a higher proportion of leafage.

I. INTRODUCTION

ASSESSMENTS of the vigour of a scion variety are, of necessity, usually made by grafting it on one or more rootstock varieties and comparing its growth with similarly worked standard varieties. Under these conditions the general level of growth will vary from rootstock to rootstock, so that such assessments of vigour can only be made in terms of the vigour of the rootstock variety, or, for a rootstock, in terms of the scion variety. Similarly, the growth of the shoots of an unworked rootstock or seedling must be assessed in terms of the growth of its root system.

A possible method of estimating scion vigour free from root restriction might be to utilize the strong stimulation in shoot growth following heavy pruning. When a tree is pruned back hard, a profusion of vigorous shoots springs from the cut stump, many of them making far more than the usual annual increment. Since prior to pruning the root system was in equilibrium with the shoot system, it should afterwards be in excess and hence not a limiting factor in growth. So, if a scion were stump grafted on to a heavily pruned tree, the resulting growth might be maximal, or unrestricted in the sense that it would be free from limitations due to the size and activity of the root system. The analogy with 'derestricted roads' is useful, since even under ideal driving conditions the speed of a car does not increase indefinitely, but becomes subject to limitations such as horse-power, variety, or passengers, which may be less critical in congested traffic. D'Arcy Thompson (1942, p. 211) has used 'unrestricted growth' for the very rapid relative growth of the claw of the male fiddler crab compared with total body-weight; but in the

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present context the important implication is that one of the restrictions normally present—due to the root system—has been removed. Whether in fact, the restriction removed was solely that imposed by the root system—water, inorganic nutrition or root syntheses (Mothes, 1955)—or whether there was in addition a positive contribution of reserves from the rootstock to the growing scion, has not been decided in these experiments, though the fact that the rootstock stem thickened considerably during the season suggests that this contribution was small and limited to the early stages of growth. This effect of pruning would only be temporary, since with the formation of new root and shoot tissues in a constant ratio (Knight, 1934) the normal root / shoot ratio for the tree would gradually be re-established. However, a pruning response persists for 2–3 years after heavy pruning, so that at least during the first year's growth, it might well be assumed that the root system was not limiting growth. This approach was therefore made in the two experiments to be described.

II. METHOD

Apples

In 1954 six 40-year-old bush apples were grafted with scions of vigorous and dwarf varieties of apple. Six branches on each tree were cut back to breast height, the remainder being judiciously trimmed as what are called 'sap-drawers'. Each stump was furnished with three oblique cleft grafts of the particular variety (Garner, 1947, p. 178). The varieties were the rootstocks M. XVI (vigorous), M. IX (dwarfing) (Hatton, 1935), and 3426 (extremely dwarfing) (Preston, 1954); two stumps per tree were worked with each variety. As a result of the heavy pruning, shoots grew out profusely from the rootstocks; most of these were rubbed out and the remaining few removed as the scion developed. Surplus scions were removed in June, and the single shoot allowed to remain was tied to canes to prevent the graft from blowing off in the wind.

An analogous experiment was set up on the root system of six adjacent trees. The nature of the material enforced some modification of the method, since a minimum of intact root system had to be left for the tree to survive. In March a circular trench was dug 2 feet deep, 3 feet away from the base of the tree, and all roots encountered cut cleanly across. The scions, collected the previous winter, were pieces of root 4–6 inches long of the same three varieties. These were cleft grafted into the split ends of stock roots $\frac{1}{4}$–$\frac{1}{2}$ inch in diameter taking care to maintain their polarity. The unions were covered with bitumastic grafting wax and bound with waterproof tape; they were then inserted into tubes of 3-inch layflat polythene containing a damp mixture of peat and sand, both ends being closed with rubber bands. Movement and bending of the grafts were prevented by tying the tubes to canes fixed across the trench. About twice as many roots were grafted as were finally required. Afterwards the trench was loosely filled in with straw to maintain a uniform dark environment. A few weeks later, new white roots could be seen in many of the tubes, and some were also showing shoots. On June 2, the grafts were established into 10-inch pots of John Innes compost. New roots developed only from the distal ends of the grafts, and shoots from the proximal ends only of those grafts which had not taken; occasionally, adventitious rootstock shoots were produced within 2–5 cm. of the cut base, irrespective of the success of the graft. The pots were inspected at intervals, and watered as necessary; on one or two occasions the pots probably became too dry.

Plums

With slight modifications the same methods were used for plums in 1955. When grafted, the rootstock trees were entering their third season; they had received a minimum of pruning and were some 2·5 m. high with stems about 5 cm. diameter at 15 cm. above soil-level. Trees for shoot grafting were cut off at 1 m., and three scions of a single variety were either side-grafted on to the stump, or whip-and-tongue grafted on to convenient laterals. Only the most vigorous was allowed to grow on. All other shoots were removed as they grew out. Root grafts were made by excavating on each side of the tree until enough suitable roots had been exposed, four being worked to each tree. No attempt was made to prune the root system heavily, as it seemed from the apple experiment that root growth was more influenced by soil volume available for growth than by pruning. Successful root grafts were later established in a thoroughly mixed unsterilized compost containing 25 per cent. sand, but no peat, as this could not be completely separated from the finely matted roots. Throughout the growing season the pots were kept adequately watered. The trees had previously formed an experiment involving the rootstock varieties Myrobalan B, Brompton, and Pershore in various combinations and the layout permitted the estimation of any rootstock effect. This effect proved negligible and will not be referred to subsequently. The scions were two cultivated varieties of plum (*Prunus domestica* L.) Warwickshire Drooper and Victoria Plum, and two wild sloes (*P. spinosa* L.) Large Sloe (accession No. E328) and Dwarf Sloe (accession No. E329). Warwickshire Drooper is considered a very vigorous scion variety and Victoria Plum vigorous; on their own roots they made similarly sized trees; the Large Sloe was about twice the size of the Dwarf Sloe.

III. RESULTS

1. *Apples 1954*

Most of the grafts took and grew well, but by late July, dead brown patches began to appear on the leaves of rapidly growing shoots: nevertheless growth was much greater than normal, though probably not maximal. A summary of the results is given in Table I.

Under normal conditions of growth, the expected order of weight would be M. XVI, M. IX, and 3426, but in the experiment, while M. XVI roots were heaviest, the heaviest shoots were 3426, which were also significantly longer and had more secondary leaves. The indication therefore was that the growth potentialities of the roots and shoots of a single variety were not necessarily the same.

TABLE I

Apples. Data on Shoot and Root Grafts

Scion variety	M. XVI	M. IX	3426	Significance
Shoot dry weight	18·7	16·2	26·9	N.S.
Shoot length	52·3	54·3	89·2	Diff. 26·7*
Number of secondary leaves .	0·1	9·7	29·1	Ratio 12·9**
Root dry weight	33·1	22·4	23·3	N.S.
Number of vigorous root grafts	6	7	3	

$*p < 0{\cdot}05$ $**p < 0{\cdot}01$

2. *Plums 1955*

(*a*) *Root growth.* For various reasons, a number of the root grafts made poor growth and since the experiment was concerned with maximal growth, scions less than the median in weight have been excluded from the data on root weight given in Table II. There was insufficient data on the Dwarf Sloe. The mean (geometric) weight of Warwickshire Drooper was much less than Victoria or Large Sloe, but owing to the overlap between the smaller individuals adequate statistical significance is only reached when the largest 3–4 individuals in each variety only are considered. It is concluded that real differences do exist in the potential growth-rates of roots of the varieties, these differences reaching maximum expression when growing conditions are very favourable.

(*b*) *Shoot growth.* Shoot growth was generally healthy; in July the youngest leaves of Warwickshire Drooper showed the minute black spots indicative, in this variety, of a transient attack of Bacterial Leaf Spot. Leaves on the primary stems of Dwarf Sloe showed a chlorophyll abnormality, most noticeable on the largest leaves; this appeared as a sharply defined patch of paler green on either side of the midrib, and proved to be due to the replacement of green by colourless mesophyll. All Dwarf Sloe shoot scions showed these symptoms, though they could not be found on the parent clone. In subsequent tests this variegation was again only produced on rapidly growing grafted shoots and was not graft-transmissible.

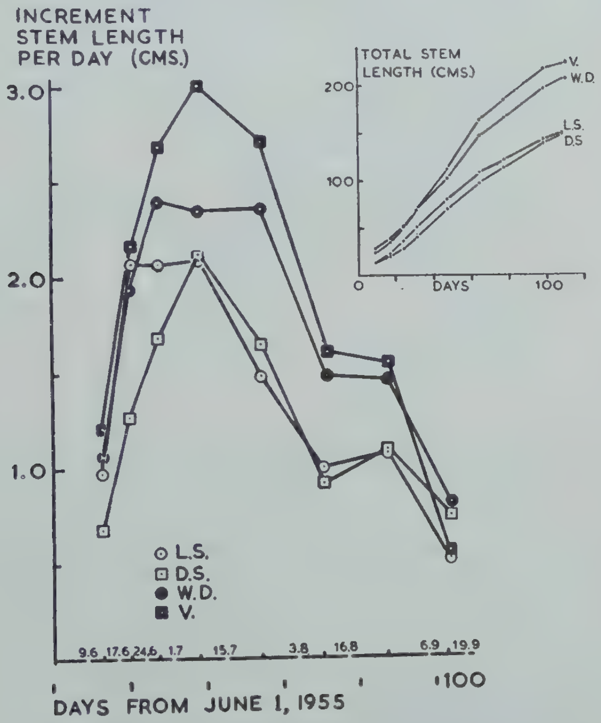
TABLE II

Dry Weight of Root Grafts (g.), 9 Nov. 1955

	Dwarf Sloe	Large Sloe	Victoria	Warwickshire Drooper
Number of grafts . . .	2	7	8	16
Geometric mean weight (above median)	—	9·9	10·6	5·0
1st, 2nd, and 3rd heaviest roots	—	13·8	26·4	8·0
	—	13·6	11·9	6·0
	—	9·6	9·6	5·4

(i) *Growth of the Primary Stem*

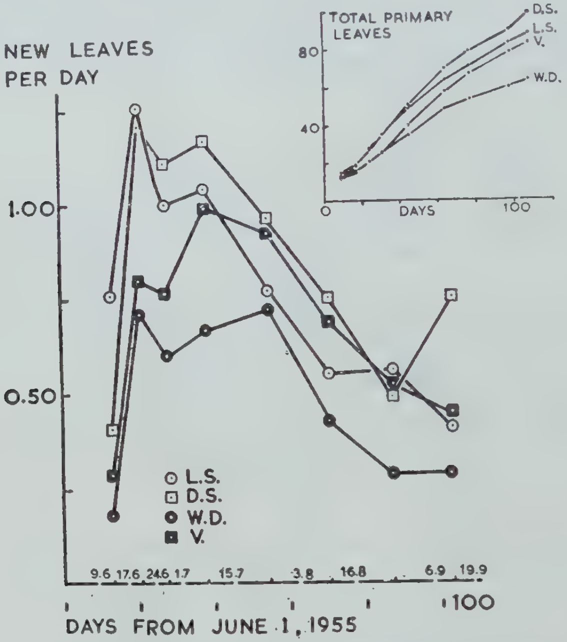
The plum shoot has a naturally rapid rate of growth, and, enhanced as this would be by the heavy pruning, any major divergences from the normal 'Grand Curve of Growth', such as might be induced by the onset of rootstock limitation, should be readily detected by fairly frequent measurements of stem length. Such measurements have been summarized in Text-fig. 1. The principal deviation from the normal Grand Curve was the resumption of more rapid growth towards the end of August, but an autumn flush of growth is not an unusual occurrence in fruit trees, and cannot here be interpreted as marking the onset of rootstock limitation of growth.



Text-fig. 1. Average daily rate of elongation of the primary stem (cm. per day). L.S. Large Sloe, D.S. Dwarf Sloe, W.D. Warwickshire Drooper, V. Victoria.

Apart from the slight lagging behind of the Dwarf Sloe due probably to the grafts taking longer to become established, the acceleration of growth to the maximum rate was the same for all varieties. However, they differed in the actual initial and maximum rates, indicating that, in the absence of rootstock limitation, there was not an upper limiting value common to all varieties, but that, as might be expected from considerations of apical organization, each variety tended towards a characteristic maximum. The maxima reached are high; for the first fortnight in July the Sloes averaged 2·1 cm. per day and Victoria Plum 3·1. Individual maxima were not much greater, as if all shoots were growing at a maximal rate; values were Dwarf Sloe, 2·4; Large Sloe, 2·5; Warwickshire Drooper and Victoria Plum, both 3·4 cm. per day. These values overlap those quoted by Oppenheimer (1945) for high growth-rates of non-climbing dicotyledons.

Rate of leaf production is not usually greatly influenced by the rootstock, and the data for primary leaf production shown in Text-fig. 2 give no evidence for the intervention of a rootstock effect.



Text-fig. 2. Average daily rate of leaf production by the primary stem. L.S. Large Sloe, D.S. Dwarf Sloe, W.D. Warwickshire Drooper, V. Victoria.

Although all varieties had a similar pattern of leaf production by the leader tip—a rapid rise and a peak period with about one new leaf per day followed by a decline—there was a marked difference between the species. In the sloes, the maximum was reached by the end of June with peak production lasting 20 days, but in the plums, the rise was more gradual and the peak lower and spread over 40 days. As the graph for total leaves shows, this resulted in an early separation of the two species. The varietal differences were also well marked both initially and subsequently, the differences building up throughout the season.

(ii) *Total Stem*

The total growth of vigorous shoots, however, consists of more than the extension of the single primary leader; in addition to varying amounts of stem thickening, secondary shoots are produced on the current season's stem, and it is these that constitute the chief mechanism by which differences in total growth can be expressed. In this experiment such effects were exaggerated, the Large Sloes even producing quaternary branches. The relations between the weights of the various parts have been examined with a view to determining how far growth has been limited by morphological relations or by, for example, leaf efficiency. Since the experiment was concerned with maximal growth, weights and stem lengths are given in Table III.

TABLE III

Stem Data, 22 Sept. 1955 (All shoots)

	Dwarf Sloe	Large Sloe	Victoria Plum	Warwickshire Drooper
(*a*) Dry weight (g.)				
Total shoot	250	295	243	483
Total stem	168	195	153	298
	Per cent. stem weight as			
Primary stem	34	29	60	52
Secondary stem	50	50	40	47
Tertiary and quaternary stem	16	21	0	1
(*b*) Stem length (cm.)				
Primary stem	151	151	218	215
Secondary stem	2,006	1,995	673	1,464
Tertiary and quaternary stem	1,565	2,244	4	38

The Warwickshire Drooper shoots were double the weight of the Victoria, thus reversing the behaviour of the roots; the Sloes were about equal in weight. Within each species the extra weight of the heavier variety was due to the higher order of branches involving a larger proportion of the stem weight. Thus, despite the much heavier primary stem of the Warwickshire Drooper, its secondary stems comprised 47 per cent. of the total stem weight as against 40 per cent. of the Victoria.

The differences between species are clearly shown by a comparison of the equally heavy Sloes and Victoria, the former with about 20 m. each of secondary and tertiary branches and the Victoria with less than 10 m. Only a little more thickening of the Sloe branches would have produced a stem weight considerably greater than that of the Victoria. It is only among trees of similar form that the amount of secondary branching is a measure of total growth.

(iii) *Final Leaf Weight and Number*

Data on leaf weight were obtained when the scions were harvested in September. The leaves were stripped in the field from the successive orders of branches and known numbers of them, obtained by thoroughly mixing and quartering the heap, were fresh weighed at the same time as the bulk of the leaves. Shed leaves were counted when the leaves were picked and have been included in the totals. Varietal averages for the various orders of branching are given in Table IV.

TABLE IV

Leaf Data, 22 Sept. 1955

Variety	Dwarf Sloe	Large Sloe	Victoria Plum	Warwick Drooper	Statistical significance p = 0·05
1	2	3	4	5	6
(*a*) Average weight of single fresh leaf (g.)					
Primary . . .	0·107	0·158	1·230	1·995	Ratio 1·15
Secondary . . .	0·066	0·091	0·676	1·000	
Tertiary . . .	0·030	0·028	0·300	0·363	
Quaternary . . .	—	0·004	—	—	
(*b*) Number of leaves					
Primary . . .	117	118	76	65	
Secondary . . .	2,038	1,755	230	412	
Tertiary . . .	1,795	3,822	2	29	
Quaternary . . .	12	12	0	0	
Total (geometric means)	3,891	4,898	282	501	Ratio 1·35
(*c*) Leaf dry weight					
Total (g.) . . .	82	100	90	185	Diff. 24
Percentage of total leaf dry weight as					
Primary . . .	8	8	40	24	
Secondary . . .	68	59	60	74	
Tertiary and quaternary	24	33	0	2	

There was clearly an inverse relation between rate of leaf production (Text-fig. 2) and single-leaf weight, Warwickshire Drooper having larger leaves than Victoria, and plums larger leaves than sloes. Leaf number was also obviously related to rate of leaf production and possibly inversely related to leaf size (Table IV*b*), being high in the sloes and lower in the plums. The number of secondary and tertiary leaves reflected the amount of branching. Leaf size and leaf number were not strictly inversely related, or the total leaf weight would be constant, which it was not (Table IV*c*). Since leaf expansion and leaf initiation are presumably quite different kinds of process, it is of interest to find whether single-leaf weight or leaf number is of greater importance in determining total leaf weight. The data for leaves on secondary shoots were examined, since these most closely reflected the size of the shoot. It was found that the total correlations of single-leaf weight or leaf number with total leaf weight, though often highly significant statistically within each variety, varied from positive to negative from variety to variety. Nor was any consistent pattern revealed when allowance was made for variation in leaf numbers or weights by calculating the partial regressions.

As with stem lengths, despite large differences in habit and leaf number, the total leaf weights of sloes and of Victoria Plum were little different, all three averaging about half the weight of Warwickshire Drooper. The plums, however, carried a much greater amount of their leafage, both absolutely and proportionately, on the main stem, while a quarter of the sloe leaves were borne on the tertiary shoots.

(iv) *Single Leaf Size* (Table IV*a*)

Within a variety, despite their nearly concurrent production, the primary leaf is about twice the weight of the secondary and the secondary thrice the weight of the tertiary. This relation between single-leaf weights of successive branch orders was evident even for individual shoots, and further examination showed that there was a statistically significant relation between the average weights of leaves on successive orders of branches (Table V). It is quite clear that the ratio of primary- to secondary-leaf weight tended to be constant at values near 2, and that the square root of the ratio of secondary to tertiary was close to the primary/secondary ratio, so that the average leaf weights on successive orders of branches were approximated by the series, a, ap, ap^3. The ratios primary/secondary and $(\text{secondary/tertiary})^{\frac{1}{2}}$ in the table give two estimates of p. The right-hand column of Table V gives the fiducial limits of the ratio of these estimates; the expected value for equality is 1, and as the limits show, most of the observed values do not differ greatly from this.

TABLE V

Ratios of Average Single-Leaf Weight on Branches of Successive Orders

Variety 1	No. Shoots 2	$\frac{\text{Primary}}{\text{Secondary}}$ R_1 3	$\sqrt{\frac{\text{Secondary}}{\text{Tertiary}}}$ R_2 4	5% Fiducial Limits of R_1/R_2 5
Dwarf Sloe . . .	10	$1{\cdot}71 \overset{\times}{\div} 1{\cdot}08$[1]	$1{\cdot}57 \overset{\times}{\div} 1{\cdot}05$[1]	0·92—1·31
Large Sloe . . .	10	$1{\cdot}84 \overset{\times}{\div} 1{\cdot}04$	$1{\cdot}94 \overset{\times}{\div} 1{\cdot}04$	0·80—1·11
Victoria . . .	9	$2{\cdot}08 \overset{\times}{\div} 1{\cdot}06$	—	—
Warwickshire Drooper .	11	$1{\cdot}97 \overset{\times}{\div} 1{\cdot}08$	1·67 (based on 5 shoots only)	—

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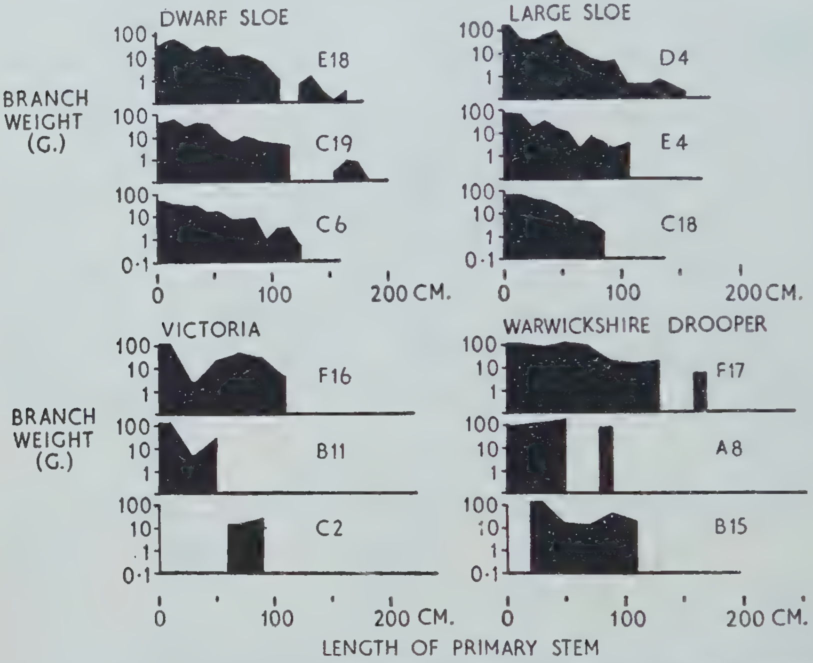
[1] Calculations based on logarithmic transformation and Standard Errors transformed back to ratios.

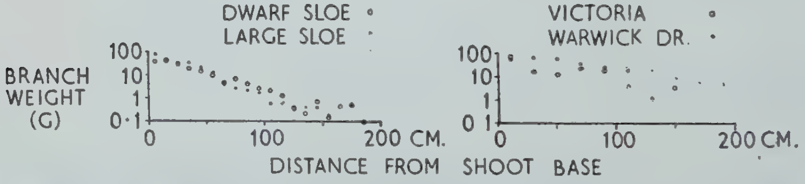
Although the leaves become more crowded (2–4 fold) on successive orders of branching while the leaves themselves become progressively smaller, leaf weight per cm. stem is not constant but decreases 2–3 fold.

(v) *Form*

The outline of the whole shoots was roughly triangular, suggesting that the growth-rate of lateral branches was proportional to that of the primary stem (Penrose, 1925). Production of laterals was not uniform, regions with short or no laterals alternating with regions with longer laterals (Plate XXI). Assuming the stems to taper uniformly, the weight of a single lateral would be proportional to the cube of its length, and since length is approximately proportional to the distance from the base of the primary stem, log. branch weight should be proportional to distance from stem base. The fresh weights of laterals borne on successive 10 cm. intervals of the main stem were determined at harvest, and plotted against stem length for individual trees. In general the result was a series of undulations whose peaks lay along a straight line sloping to the apex of the shoot; this was most clearly shown by the sloes, the valleys for the plums often being zero. Text-fig. 3*a* shows the curves of the largest, median, and intermediate shoots in each variety. When all shoots of a single variety were grouped, the heights of the peaks were still fairly strictly related to distance along the shoots (possibly because of the limited range in length of the primary stem) and the averages of the log. branch weights lay



TEXT-FIG. 3*a*. Specimen histograms relating branch weight to distance of the branches from the base of the primary stem. The branches over 10 cm. primary stem (sloes) or 20 cm. (plums) were weighed as a group.



TEXT-FIG. 3*b*. Mean weight of branches borne on successive intervals of the primary stem (geometric mean). Only branches weighing 0·1 g. or more have been averaged.

along a somewhat sinuous line sloping to the tip (Text-fig. 3*b*). The individual curves confirm the visual impression that the unit of secondary-shoot growth was not the single shoot, but the shoots over about twelve primary leaves. In the plums there was considerable inhibition of laterals, whole units of secondaries being absent. As the average curves show, where laterals have developed, their weight was what would be expected from their position on the shoot, and not the sum of the hypothetical weights of all the missing shoots. It may be that this inhibition of the growth of lateral shoots was limiting total shoot weight, since if the limitation were due solely to lack of anabolites, the primary stem should be furnished along its length with a complete series of diminished laterals, whereas the evidence suggests that if an inhibited set of shoots could be induced to grow out, it would have a weight characteristic of its position. Further, the heaviest shoots were those most completely furnished with laterals, or as in the sloes, laterals on laterals.

IV. Discussion

1. *Growth Potentialities of Roots and Shoots*

These experiments showed that the unrestricted growth-rates of the root systems of the varieties used did not run in the same order as the growth-rates of the shoot system. Moreover, whereas the *shoots* of Warwickshire Drooper were twice the weight of the Victoria shoots, the Warwickshire Drooper *roots* were only half the weight of the Victoria roots. Since the parent trees from which the graft material came were of the same size, these observations fit Vyvyan's view that the growth-rate of the compound tree is the resultant of the growth-rates of the component varieties (Vyvyan, 1955). Hatton's data (1936) indicate that own-rooted Warwickshire Drooper and Victoria make similarly sized trees. Skinner (1952) also has shown that cultured roots excised from apparently similar sized plants of *Senecio vulgaris* grew at very different rates. This would suggest that there is no necessary correlation between the *potential* growth-rates of the root and shoot systems of a plant on its own roots—a point which may be important in selecting rootstocks since a potentially dwarfing root system could be masked by a vigorous shoot system or vice versa. In fact Hatton (loc. cit.) and Kasjjanenko (1950) comment on the vigour of Victoria on its own roots and Glenn (1956) considers it a possible vigorous rootstock.

This type of rootstock-scion interaction must be distinguished from the cases described by de Stigter (1956) and Dotti (1955) where the scion variety apparently fails to fulfil the nutritional requirements of the rootstock, which eventually dies. In these plums the varietal differences were produced in root or shoot systems growing under conditions of abnormally high nutrition.

2. *Efficiency of Shoot Growth*

A comparison of the sloes and plums shows that total shoot weight is the resultant of the thickening of stems and expansion of leaves rather than of the rate at which new leaves and shoots are initiated. The slower growth of the sloes was not due to delay in producing secondaries until late in the season, since on June 8 the average number and length of secondary shoots was Dwarf Sloe, 7 shoots on 17 cm. primary stem, Large Sloe, 9 on 18 cm., Victoria, 3 on 23 cm., and Warwickshire Drooper, 5 on 31 cm. primary stem, and as has been shown, production of secondary shoots was closely related to stem extension, which followed a similar course for all varieties.

An indication of the efficiency of the foliage in producing the total shoot weight was obtained from the stem/leaf ratios. Although differences within *species* were not significant, in each case the variety with the higher proportion of leaf made the larger total shoot. Within each variety the trend was reversed, the heavier shoots having a smaller proportion of leaf than the lighter. The correlation between shoot weight and stem/leaf ratio fell just short of statistical significance, being 0·332 ($r = 0{\cdot}349$ for $p = 0{\cdot}05$) for the average trend *within* varieties, and −0·725 ($r = -0{\cdot}878$ for $p = 0{\cdot}05$) *between* varieties. Within individual varieties the correlation was significant for Dwarf Sloes, and just short of significance for the Large Sloes, which latter had a much steeper regression line; the plums showed no significant regression. Despite the uncertainty in the detailed results, there is little doubt of the existence of opposing trends between and within varieties.

As shown below, this result would be expected from the postulates that, under conditions of unrestricted growth, the anabolic activity of the foliage of the different varieties was the same, and that under these conditions the differing final dry weights of the varieties were produced by differential partitioning of the anabolites between stem and new leaf. Where growth was restricted—though still vigorous—so that the output of the leaves was impaired, total growth would be reduced and this could lead directly to lowered total weight with constant stem/leaf ratio, as within plum varieties, or, where efficiency was so impaired as to reduce the amount deposited as secondary thickening, to a reduced stem/leaf ratio, as within sloe varieties.

If, on the other hand, it is assumed that the excess vigour resulted from translocated rootstock reserves, a more complicated situation arises. Firstly, this 'unearned income' may have been available only in the early stages of scion growth, the subsequent rapid growth being homeostatically maintained. In this case the previous argument applies unchanged. Secondly, the rootstock reserves may have been absorbed continuously throughout the growth of the scion. Anabolic material would then be available to the shoot from two sources—rootstock and leaves—and would be utilizable in stem or leaf. Within the variety, where the heavier shoots have relatively more stem than have the lighter shoots, the increased weight could result from the stem acting as a more capacious 'sink' for anabolic materials than the leaves. Between varieties, where more vigorous varieties have more leaf, a uniform anabolic rate with or without differing rootstock contributions may be assumed.

These conclusions are formally verified below. The anabolic rate of the scion shoot for this purpose is defined as the increment in weight of the shoot per unit of leaf per unit time. It is therefore identical with Net Assimilation

Rate and Unit Leaf Rate (Gregory, 1926; West, Briggs and Kidd, 1920) except that the rootstock—trunk and roots—is ignored. The rate may vary over short periods, but as with the N.A.R., the average value over a longer period may be calculated (Williams, 1946; Fisher, 1921).

If a scion, shortly after unfolding its leaves, has weight G_0 comprised of stem S_0 and leaves L_0, then the increment, g_0, over the ensuing short period is $g_0 = aL_0$, 'a' being the anabolic rate. This increment is composed of leaf and stem in the ratio $p:q$ where $p+q = 1$, so that at the end of the period the leaf weight has become $L_1 = L_0+pG_0 = L_0\,(1+pa)$. By repeating this process, the weights of stem, leaf, and shoot over a number of periods may be found. The final leaf/stem ratio is found to be independent of the anabolic rate of the leaf and if the initial weights are negligible compared with the final weights, it equals p/q. By making the periods very short and numerous, the average value, A, of the anabolic rate may be derived from the limiting values

$$G_n = \frac{Lo}{p}.e^{Atp} \text{ or } L_n = L_0.e^{Atp}$$

and is virtually the same as the Net Assimilation Rate. Thus while the stem/leaf ratio is independent of the anabolic rate, the final weight of the shoot is dependent both on anabolic rate and leaf/stem ratio. By assuming that the initial weight is negligible compared with the final weight

$$A\,(t_2-t_1) = \frac{1}{p}(\log L_2 - \log L_1)$$

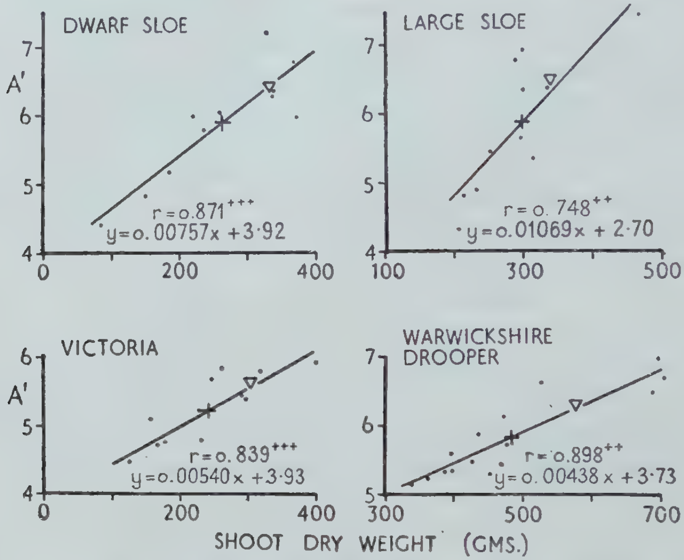
or, since the absolute value of A is not important here,

$$A' = \frac{\log L_2}{p}.$$

The varietal regressions between A' and shoot weight have been plotted in Text-fig. 4. All lines are statistically highly significant, and confirm the postulates made earlier—that the activity of the leaf was lower for lighter shoots, and that for the largest shoots varietal differences in A' were small. It may be noted that an S curve can be readily fitted to the sets of points, indicating an upper limiting value for A'.

As has been shown, the form of the trees was such that it could justifiably be assumed that growth followed a regular pattern during the season, and even if the final stem/leaf ratio differed from earlier values, the time trend of the ratio was nearly the same for all the individuals of a variety. Consequently, the differences in calculated anabolic rate represent real differences during growth, and are not artefacts inherent in the method of calculation.

Thus the anabolic activity of the leaf under the conditions of this experiment, and presumably, the Net Assimilation Rate of whole plants in comparable circumstances, depended only on the varietal average and the rate at which activity fell off under sub-optimal conditions. These arguments and results show considerable resemblance to Bald's (1946) scheme for the development of the potato plant. He assumed that 'the efficiency of the metabolic centres is similar for different varieties of the domestic potato' and that 'under similar environmental conditions the division of metabolites in various proportions between the organs of the potato plant induces in different varieties and strains differences in growth-form, maturity and yield'. These assumptions, together with the onset and duration of the various stages of growth provided a classification agreeing with the main taxonomic groups of potato varieties. However, a later paper (Bald and Hutton, 1950) suggests that the effect of infection with leaf-roll virus is to reduce the relative growth-rate but not the efficiency per unit leaf area in producing tubers.



TEXT-FIG. 4. Relation between the average increment in scion weight per gram of leaf (*A'*) and total scion dry weight.
+ general mean; ∇ mean of median and larger shoots.

In cases where the anabolic activity has been lowered, it does not necessarily follow that the efficiency of the general photo-synthetic machinery itself has been lowered; it seems more probable that the anabolic rate has been constrained from without, as by imbalance or lack of metabolites coming to the leaf, or lack of light on the interior leaves, &c.

Alternatively the reduced efficiency may be due to the suppression of laterals and consequent lack of centres of utilization for the anabolic products. Although one has a natural inclination to regard the growing-out of current season's side buds as a consequence of vigorous growth, the vigorous growth may rather be the result of the removal of the inhibition of the laterals on a plant possessing a root system capable of maintaining adequate mineral nutrition. Barlow and Hancock (1956) attribute the role of the rapidly enlarging leaf in inhibiting outgrowth of the lateral buds at the tip of the shoot to its production of auxin, so that mechanisms leading to the accumulation of such a substance in the young leaf may well play a controlling part in the maintenance of high growth-rates.

SUMMARY

1. High growth-rates of root and shoot scions of three varieties of apple (*Malus sylvestris* (L.) Mill), two of plum (*Prunus domestica* L.) and two of sloe (*Prunus spinosa* L.) were attained by grafting on to heavily pruned large rootstocks.
2. For apples, the order of final shoot dry weight was 3426, M. XVI, M. IX and of final root dry weight M. XVI, 3426, M. IX; for the Prunus species, the order was, shoots—Warwickshire Drooper, Large Sloe, Dwarf Sloe, Victoria Plum, and roots—Victoria Plum, Large Sloe, Warwickshire Drooper (there were insufficient roots of Dwarf Sloe). The largest scions of each variety differed most.
3. Warwickshire Drooper and Victoria Plum on their own roots make trees of equal size; shoot scions of Warwickshire Drooper were twice the weight of Victoria Plum, while root scions were half the weight. The results are thus in accordance with Vyvyan's hypothesis that the growth-rate of a compound tree is the resultant of the growth-rates of the component varieties.
4. For all varieties, the rates of stem elongation or of leaf production followed a similar pattern during the season, final differences being due to differences in the onset and duration of the maxima. The length of the primary stem of the plums was considerably greater than that of the sloes, and Warwickshire Drooper produced fewer primary leaves than the other varieties.
5. The average primary leaf tends to be twice the weight of the secondary leaf, which in turn is three to four times the weight of the tertiary leaf.
6. The lengths and weights of the side branches followed a rhythmic pattern along the main stem, reaching a peak value and then decreasing, followed by another peak value, and so on. The interval between peaks was about twelve leaves. The logarithm of the weight of groups of side branches over 10 cm. (sloes) and 20 cm. (plums) intervals of primary stem was proportional to their distance from the apex of the primary stem, irrespective of whether other groups of side branches had grown out.
7. The average increment in shoot weight per gram of leaf over the season tended to be the same in the fastest growing individuals of all varieties, differences in final weight being due to the higher proportion of leaf on the heavier varieties. Within varieties, the lighter individuals had lessened increments per gram of leaf, although they had the same (plums) or an even greater (sloes) proportion of leaf than the heavier individuals.

ACKNOWLEDGEMENTS

I wish to acknowledge the help with the mathematics of anabolic activity given by my colleague Mr. G. M. Jolly.

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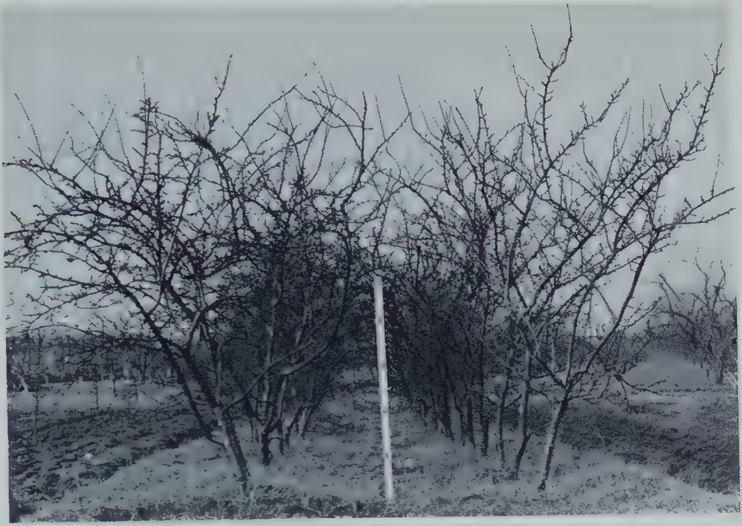
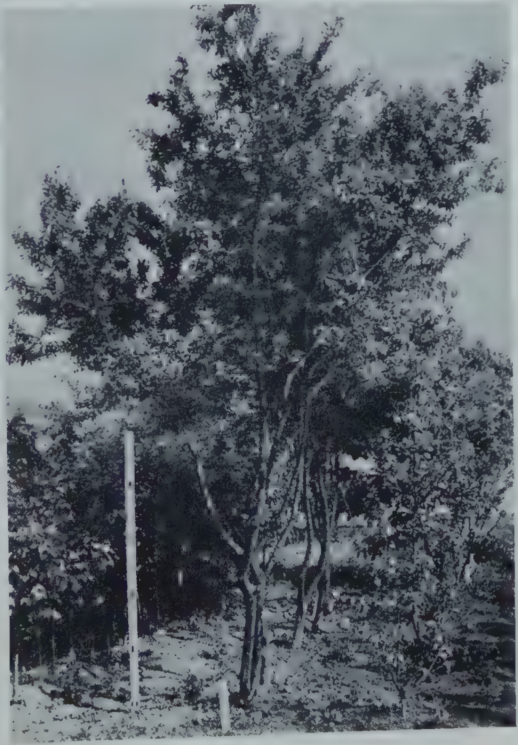
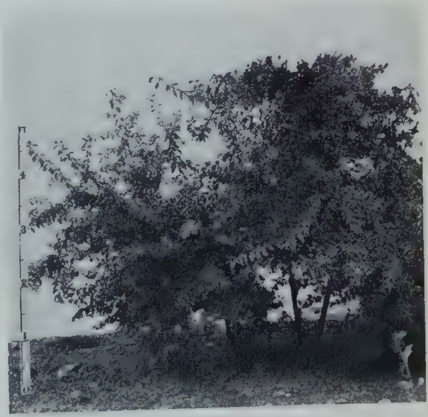


a. Warwickshire Drooper (left) and Victoria Plum (right), 7 years old



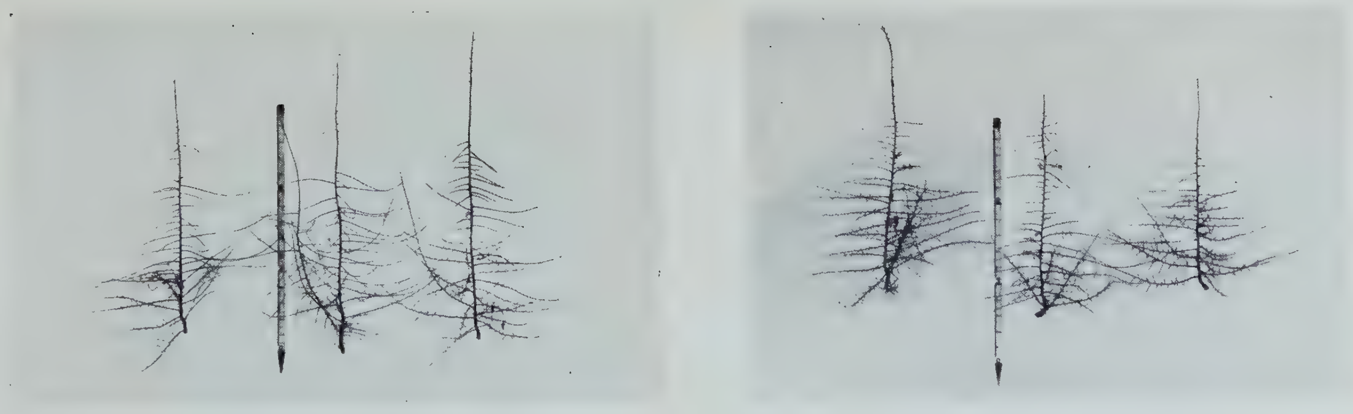
b. Large Sloe E 328, 8 years old



c. Dwarf Sloe E 329, 8 years old

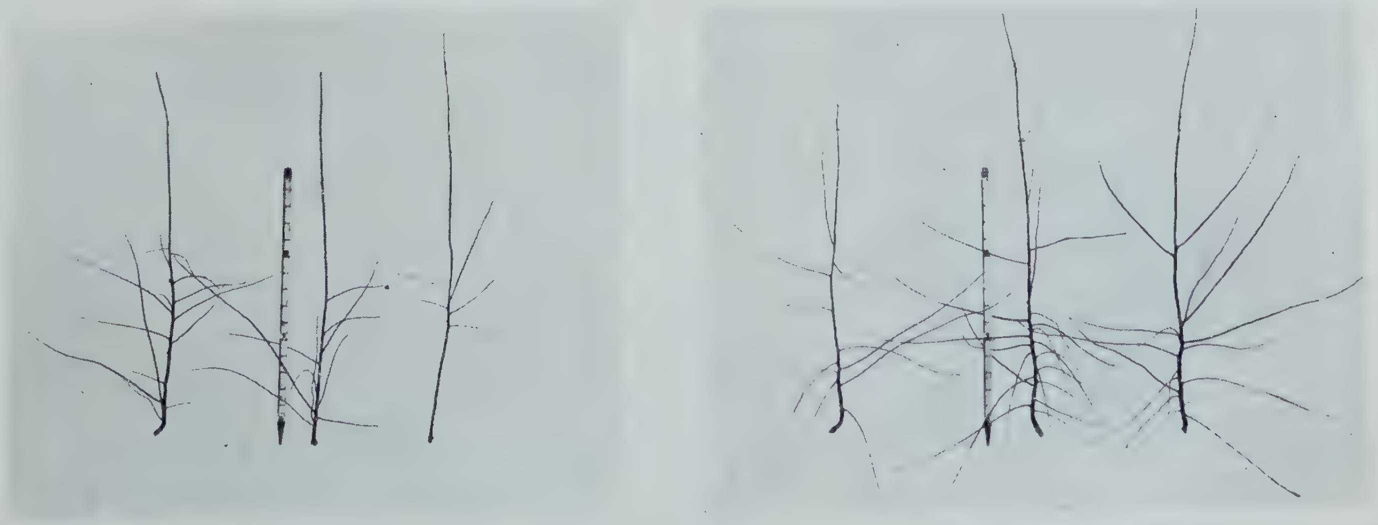
The varieties growing on their own roots

D. H. MAGGS



a. Large Sloe, E 328

b. Dwarf Sloe, E 329



c. Victoria Plum

d. Warwickshire Drooper

Scions after one season's growth. Largest, median, and intermediate between largest and median. The scale is graduated in decimetres

D. H. MAGGS

Fungal Associations

I. Synergistic Relation between *Rhizoctonia solani* Kühn and *Fusarium solani* Snyder and Hansen in causing a Potato Tuber Rot

BY

HUSSEIN ELAROSI

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With Plates XXII and XXIII and nine Figures in the Text

ABSTRACT

Rhizoctonia solani, *Fusarium solani*, and *Phoma foveata* were chosen for the study of disease caused by these fungi in different combinations in potato tubers. An initial *Rhizoctonia* infection, when followed by a *Fusarium* infection, gave an extensive rotting with external pimple-like formations in some cases. This type of rotting could not be brought about by individual infections with either of the two fungi, or jointly by them when *Fusarium* was inoculated first. Microscopic observations of infected mature and young potato tubers showed that *Rhizoctonia* grew intracellularly when infected alone, whereas it grew inter- as well as intracellularly in the successive double infection. *Fusarium* formed more haustorium-like structures when inoculated alone that when it followed *Rhizoctonia*. The length of these structures in the double infection was greater in mature than in young tubers. Atmospheric humidity affected the amount of rotting, the shape and colour of the rot, and the morphology of the fungus in the tissue.

INTRODUCTION

As Fawcett (1931) has said, Nature does not work with pure cultures, but nearly all living organisms exemplify relationships of one kind or another with other living organisms. The subject of the association of micro-organisms has been tackled from different points of view, and has been reviewed on several occasions (Machacek, 1928; Fawcett, 1931; Waksman, 1937; Weindling, 1938; Porter and Carter, 1938). The terminology of different associations has been considered by de Bary (1879), Ward (1899), Holman (1928), Waksman (1937), and Clarke (1954). The effect of phytopathogenic microbial associations on plant diseases has been discussed by Wolf (1916), Bisby (1919), Machacek (1928), Fawcett (1923), and Gemmell (1939).

MATERIALS AND METHODS

Potato tubers used throughout were of the King Edward and Majestic varieties. Healthy tubers, with a minimum of injury, were surface-sterilized with 2 per cent. formalin for $\frac{1}{2}$ minute on being received. Inoculations were made in holes 4–5 mm. diameter and 3 mm. deep in young tubers, and 5 mm. deep in mature ones.

[Annals of Botany, N.S. Vol. XXI, No. 84, 1957.]

The fungi used, *Rhizoctonia solani* Kühn, *Fusarium solani* Snyder and Hansen, and *Phoma foveata* Foister, were isolated during this work from pit rots of potato tubers.

Parasitized tissues were fixed in chromacetic acid, and embedded in wax for detailed examination. Stoughton's (1930) method for the differentiation of bacteria and fungi in plant tissues was used. With this stain the cell walls of healthy tissues took up the orange-G colour, while the thickened cell walls in the region of fungal invasion stained green; starch grains stained blue. *Fusarium* hyphae were a faint yellow with blue haustoria. *Rhizoctonia* hyphae were originally brown in colour. Suberin and suberized cells were examined by a modification, devised by the author, of Artschwager's (1927) method, in which orange-G was used as a contrast stain to the ammoniacal gentian violet. Colours of rotting tissues were named by reference to Ridgway's (1912) colour standards.

EXPERIMENTAL RESULTS

(*a*) *Relation of an initial to a subsequent infection*

At an early stage in the investigation of potato tuber rotting by fungi, the results obtained suggested that when two species were inoculated one after the other in the same locus, the actual inoculation sequence had an effect upon the extent of the rotting produced. This observation was made the basis of further intensive investigations described below.

Tubers were inoculated in two sites, one near the apical or rose-end and the other near the stem-end. Each site received one inoculum, and then, after a month, a second inoculum. Tubers were examined two months after the first inoculation. Treatments 18 to 20 (Table I) consisted of wounding and storing for one month, then inoculating in the same position as in other treatments. Controls no. 4 and 8 were for tubers wounded once. Control no. 21 was for tubers wounded twice in the same position after an interval of one month. The amounts of rotting obtained from each treatment (3 tubers) are given in Table I. The least significant differences were found to be 4·04 at 5 per cent. probability and 5·31 at 1 per cent.

Tubers inoculated initially with *Rhizoctonia*, and then, after a month, with *Fusarium*, gave the maximum amount of rotting in the stem-ends of both varieties, these figures being significantly greater than those from any other treatment. The extent and kind of rot produced was characteristic of each fungus. Thus, with *Rhizoctonia solani*, the periderm round the inocula became raised, hardened, and cracked radially from the inoculum. The rot had the typical dry core (Ramsey, 1917), 1 to 3 mm. in diameter, which was surrounded by 1 to 2 mm. of 'burnt umber' coloured tissue. In most cases the infection extended in two directions, taking a T shape, and causing surface and deep rotting. It was also observed that the dry cores tended to extend towards the rose-end. *Fusarium solani* produced limited lesions, funnel-shaped in most cases, 'dusky brown' in colour, surrounded by a thin margin of 'dresden brown' tissue. No external symptoms were observed. When

Phoma foveata was used, the symptoms were characteristic of the 'gangrene' disease (Whitehead, McIntosh, and Findlay, 1945). This rot could be recognized externally by a depression of the surface with concentric rings of corrugations round the inoculum. The depression became oval in shape in the long axis of the tuber. Rots typically extended to the centre of the tubers rather than to the sides. The colouring of the rot appeared in zones as follows: the healthy tissue was followed directly by a narrow margin (1 to 3 mm. deep) of 'mars brown' coloured tissue, then a wide zone of dry, loose starchy tissue, 'light buff' in colour, which surrounded a cavity, where, in some cases, white-grey mycelium of the fungus appeared.

TABLE I

Effect of Inoculation on the Amount of Rotting (gm.)

	King Edward		Majestic		Total
	Stem-end	Rose-end	Stem-end	Rose-end	
1. *R. solani* after 1 month	0·50	0·54	0·59	0·52	2·15
2. *F. solani* ,, ,, ,,	0·31	0·38	0·48	0·34	1·51
3. *P. foveata* ,, ,, ,,	1·92	1·68	1·55	1·37	6·52
4. control ,, ,, ,,	0·06	0·06	0·06	0·06	0·24
5. *R. solani* after 2 months	0·56	0·59	0·75	0·53	2·43
6. *F. solani* ,, ,, ,,	0·58	0·58	0·57	0·51	2·24
7. *P. foveata* ,, ,, ,,	2·07	1·75	1·76	1·22	6·80
8. control ,, ,, ,,	0·06	0·06	0·06	0·06	0·24
9. *R. solani* then *R. solani*	0·71	0·80	0·70	0·53	2·74
10. ,, ,, *F. solani*	8·26	1·66	14·64	1·49	26·05
11. ,, ,, *P. foveata*	0·69	0·81	0·84	0·60	2·94
12. *F. solani* ,, *F. solani*	0·66	0·73	0·54	0·50	2·43
13. ,, ,, *R. solani*	0·78	0·71	0·80	0·82	3·11
14. ,, ,, *P. foveata*	1·03	0·89	0·83	1·02	3·77
15. *P. foveata* ,, *P. foveata*	3·92	2·83	1·66	2·15	10·56
16. ,, ,, *R. solani*	3·76	3·31	2·08	1·69	10·84
17. ,, ,, *F. solani*	1·36	1·62	1·31	1·44	5·73
18. 1 month storage then *R. solani*	0·55	0·45	0·69	0·45	2·14
19. ,, ,, *F. solani*	0·73	0·94	0·59	0·82	3·08
20. ,, ,, *P. foveata*	1·38	0·64	1·53	1·27	4·82
21. Control ,,	0·09	0·09	0·09	0·09	0·36
Total	29·98	21·12	32·12	17·48	100·70

In the double infections the following points were observed:

(1) *Fusarium solani*, when following *Rhizoctonia solani*, caused a marked change in the shape, as well as in the amount of rotted tissue. The subsequent lesions included two kinds of tissue: one was 'salmon-buff' and filled with small cavities; the other consisted of compact dark brown cores. The former was predominant and can be considered as the matrix of the rot, the latter being embedded at random within it. In progressive rots the compact dark brown cores were seen to be located internally to the periderm and, on the healthy surface of the infected tubers, numerous pimple-like outgrowths appeared.

(2) *Phoma foveata*, when following *Rhizoctonia solani*, did not cause any change in symptoms.

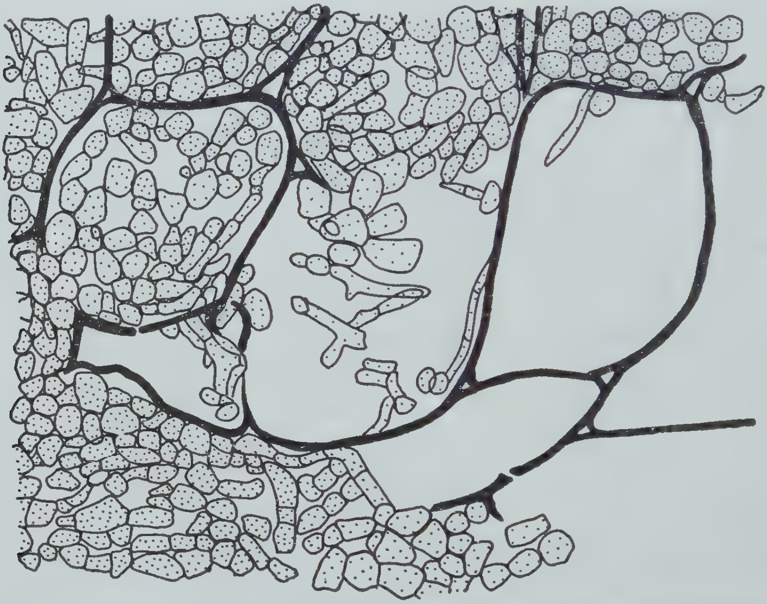
(3) *Rhizoctonia solani*, when following *Fusarium solani*, did not cause any change of the earlier symptoms.

(4) *Phoma foveata*, when following *Fusarium solani*, gave somewhat bigger lesions; the symptoms were the same as before although in a few cases the symptoms changed to those characteristic of *Phoma* infection.

(5) When *Rhizoctonia solani* followed *Phoma foveata* the symptoms of the *Phoma* infection did not change, but rotting increased.

(6) *Fusarium solani*, when following *Phoma foveata*, did not cause any change in symptoms.

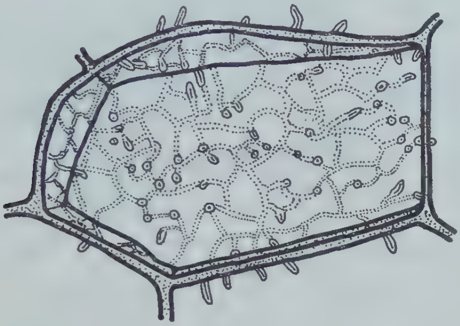
Microscopic observations: Only *Rhizoctonia* and *Fusarium* infections and their associations are considered here.



TEXT-FIG. 1. Camera lucida drawing for potato cells infected with *Rhizoctonia solani* (×250).

(1) *Rhizoctonia* alone. The fungus grows abundantly inside the host cells, crowded, thick, short cells characteristic of its sclerotia being formed. The parasite does not pass from one cell to another until the first cell becomes filled with mycelium. Thus the fungus passes through the cell walls, which appear to be disrupted under the pressure of the accumulated hyphae (Text-fig. 1 and Pl. XXII, Fig. 1). Cork layers are rarely formed, but a starch-free region of non-infected tissue was observed in the region between diseased and healthy tissues. The fungus also grows externally from the inoculum and extends outwards to different parts of the periderm, forming 'black scurfs'. These are formed of sclerotia of regular form, varying in diameter from less than 1 mm. in thickness. Some of the hyphae coming from these sclerotia attacked the sprouts and killed them.

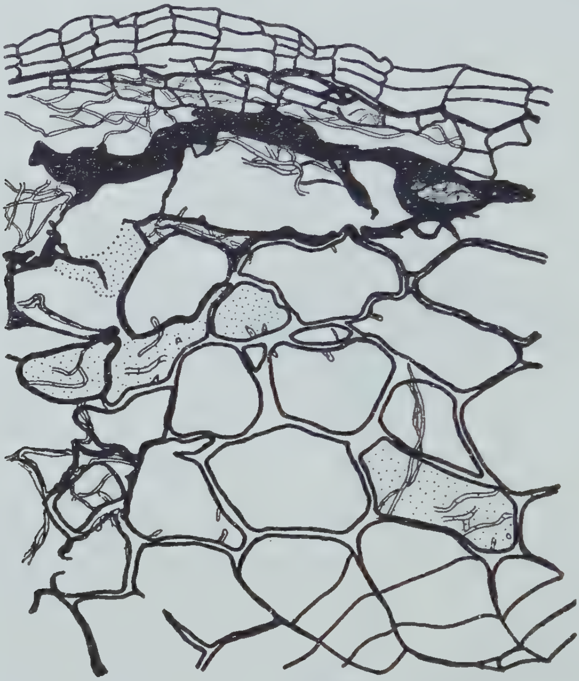
(2) *Fusarium* alone. The fungus grows mainly in the intercellular spaces, sending haustorium-like structures inside the cells, but instances of intercellular growth were observed. The hyphae of the fungus are attenuated, feeble, and stain faintly; and hence they were difficult to trace, especially in the intercellular spaces, though their path could be recognized by the haustorium-like structures (which stained blue with thionin, and reacted with ammoniacal gentian violet). The haustoria-like structures (Pl. XXII, Fig. 2) consisted of small tubes 8 to 14μ in length, and 3 to 4μ in width. In the middle of each a dark tube-like formation stops short of the distal-end, and neither takes the thionin nor the ammoniacal gentian violet stains. These resemble the haustoria of *Phytophthora infestans* (Blackwell 1953), in which the real haustoria were demonstrated to be the dark tube-like formations, the other, outer parts being considered to be the thickenings deposited by the host. Where cell walls appear in surface view, the mycelium has the appearance of a network, from which the haustoria-like structures arise (Text-fig. 2). In some cases a black band was formed a short distance from the outside cork layers. These bands are mostly caused by the aggregation of cell walls and mycelium (Text-fig. 3). Ammoniacal gentian violet enables one to see that the disease was restricted by suberin deposits as well as by the formation of cork layers in front of the advancing fungus (Text-fig. 3).



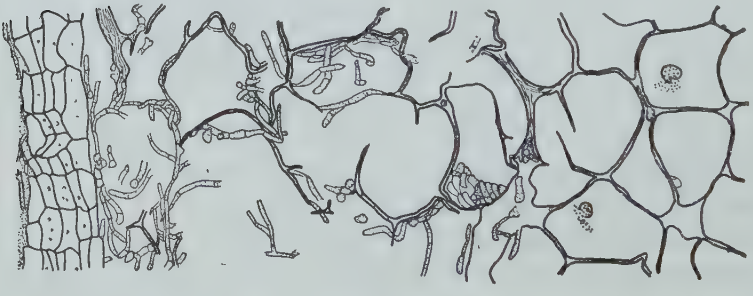
TEXT-FIG. 2. Camera lucida drawing of a surface view of a cell showing the haustorium-like structures of *Fusarium solani* and the path of hyphae in intercellular spaces (× 250).

(3) *Rhizoctonia* followed by *Fusarium*. *Rhizoctonia* was found to be the prevalent organism in the sections. It appeared in the 'salmon-buff' coloured tissue as typical elongated hyphae; and fewer sclerotia were observed than in infection by *Rhizoctonia* alone. Also, the cells were not crowded with hyphae but growth continued from one cell to the other without an accumulation of hyphae in successively invaded cells (Text-fig. 4). Sometimes *Rhizoctonia* appeared in the intercellular spaces following *Fusarium*, which grows there with rare haustorial formation.

The compact dark brown cores in the rot were composed of sclerotial cells. These had been formed inside in large numbers, accompanied by a gradual disintegration of the host cell walls. Pl. XXII, Fig. 3 shows that, in the middle of the core, host cell walls are virtually absent, whereas the host tissue at the edge of the core is still intact and coherent.



TEXT-FIG. 3. Section in potato tuber infected with *Fusarium solani*, showing dark line and cork layer formation (× 100).



TEXT-FIG. 4. Camera lucida drawing of potato tuber cells infected with *Rhizoctonia solani* then *Fusarium solani* (× 100).

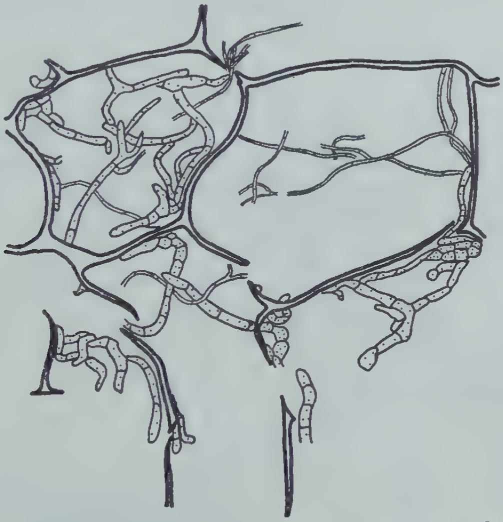
(4) *Fusarium* followed by *Rhizoctonia*. Sections showed no differences from those of *Fusarium* infection alone, except that in few cases *Rhizoctonia* hyphae were observed in the outermost cells.

(*b*) *Associations of* Rhizoctonia solani *and* Fusarium solani *in young tubers*

Young surface-sterilized tubers of the new crop (April 1955) of the King

Edward variety from the Canary Islands were used. Two wounds were made at opposite sides, half-way between the two ends of the tuber. The second inoculations were made 5 days after the first. One set of tubers was dried to 1 per cent. loss of weight by keeping under vacuum for 3 days: these were then returned to the normal incubation conditions. The second set was kept under normal conditions throughout the experiment. Controls were made by inoculating in the reverse order. Tubers were examined after a month from the first inoculation.

In the two treatments and their controls no external sign of rotting appeared, except that in all the dried tubers numerous 'pimples' appeared (Pl. XXIII, Fig. 4). When these tubers were cut into halves, it was found that nearly all the inside tissue was rotted (Pl. XXIII, Fig. 5). Infection was less in the second set. In the controls, small restricted lesions were formed (Pl. XXIII, Fig. 6).

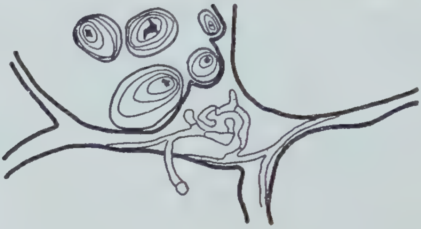


TEXT-FIG. 5. *Rhizoctonia solani* and *Fusarium solani* in the same cell of young potato tuber (×250).

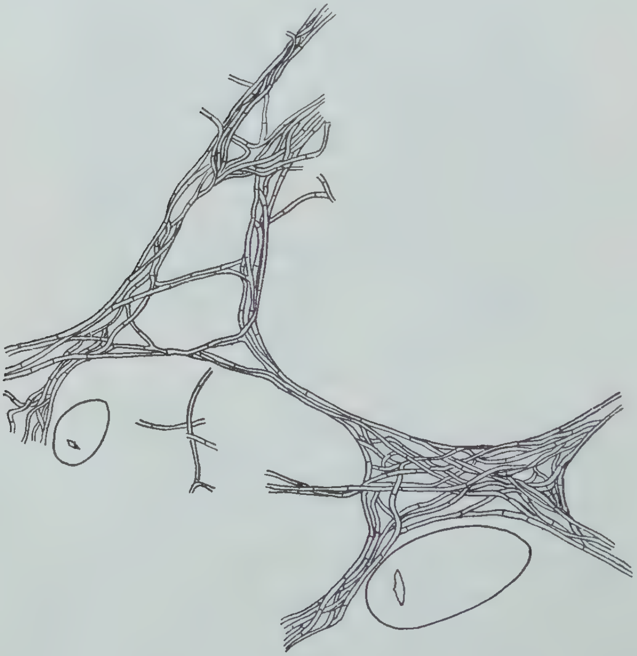
Sections were cut from the treatments of the second set to see if there was any difference between infection of young tubers and of mature ones. In the young tubers, sclerotia of *Rhizoctonia* were observed less commonly; typical elongated hyphae occurred more frequently than in mature tubers. Also, the inner parenchymatous tissue were more easily infected than the tissues near the periderm. Here *Fusarium* grew intra- as well as intercellularly (Text-fig. 5). Few haustorium-like structures were observed, and these were smaller in length (mostly between 4 to 6μ in length) than those observed in mature tubers. Unthickened structures which stained faint yellow and were less in diameter than the thickened ones were also observed (Text-fig. 6).

Pimples were examined and have been illustrated and discussed in an earlier communication (Elarosi, 1956).

In the controls of the tubers that had not been dried, it was observed that some of the parenchyma cells near the cavity formed by the cork borer had been completely disintegrated and only *Fusarium* hyphae aggregated together gave the shape of the disintegrating cells (Text-fig. 7).



Text-fig. 6. Intercellular hyphae and unthickened haustorium-like structures of *Fusarium solani* in young potato tuber (× 500).



Text-fig. 7. *Fusarium solani* hyphae forming a pattern in the place of cell walls in the border of the cavity infected with *Fusarium solani* followed by *Rhizoctonia solani* (× 375).

(c) *The effect of atmospheric humidity on the infection and progress of disease caused by* Rhizoctonia solani *followed by* Fusarium solani

King Edward variety was used. Each tuber received inocula at three different positions: rose-end, stem-end, and half-way between the two. Each site received two inocula as in the previous experiment. The tubers of each treatment were placed in a 6-inch diameter desiccator, the lower part of which contained 350 ml. of the solution or material used for adjusting the humidity. Each desiccator contained a small dish of soda lime, to absorb the carbon dioxide, and had an opening in its lid connected to a tube half-filled with the humidity-adjusting material, thus allowing of replacement by air of the carbon dioxide absorbed. A calibrated watch hygrometer placed in the desiccator gave an approximate indication of the humidity. The figures in Table II show that, in fact, the relative humidities, except in case of 100 per cent. R.H., were always much higher than those expected according to Hodgman (1948). Nevertheless, the experiment did afford a range in R.H. and incubation under these conditions gave results with statistically significant differences. Most rotting occurred at 94 per cent. R.H. in the rose-end, and in 94 to 100 per cent. in the stem-end and in the half-way position. Least rotting occurred at 68 per cent. R.H., but there was no significant difference between 68 and 81 per cent. R.H. In general, the site of inoculation does not seem to have had a big effect on infection, under the conditions of the experiment.

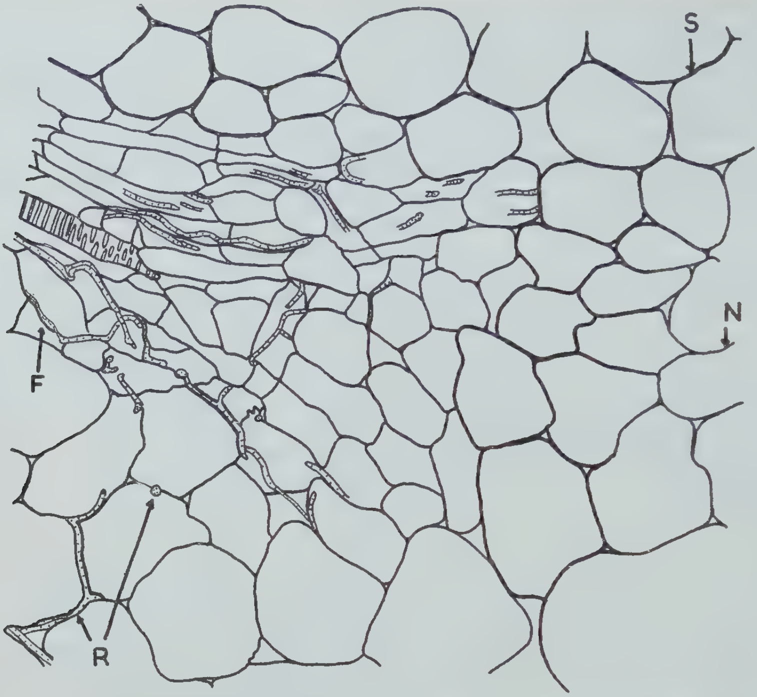
TABLE II

Effect of Atmospheric Humidity on the Amount of Rot in Potato Tubers

	Expected humidity	Observed h. (1st 10 days)	Average amount of rot (gm.)		
			Rose-end	Stem-end	Half-way
1. $CaCl_2$ (salt) . . .	0	68	0·14	0·13	0·16
2. Saturated solution of $CaCl_2$	32	90	1·01	1·60	1·28
3. 30% H_2SO_4, 1·84 S.G. .	50	81	0·65	0·59	0·86
4. 20% ,, ,,	75	94	3·04	3·17	3·37
5. Distilled water . .	100	100	1·65	3·21	2·60
L.S.D.	—	—	0·84	0·59	1·18

Atmospheric humidity not only affects the amount of rotting, but also the shape and colour of the rot. The relationships between the fungi and the tissue were also affected. In treatment no. 1, infection was not established, and wound cork layers were formed. In treatment 2, the rot was dry 'cream buff' in colour, and dark brown sclerotia were scattered in the rotted tissue. The inoculation sites were surrounded in some parts by irregular numbers of suberized cells, sometimes as many as fifteen layers. Most of the sites were invaded with *Rhizoctonia*. However, *Fusarium* was observed less frequently. Round the vascular tissue there were irregular layers of suberized parenchyma, varying from 2 to 6 layers of cells, situated above the outer phloem region (Text-fig. 8). Fungal invasion occurred in the vascular tissue as well as in the surrounding parenchyma.

In treatments 3 and 4 soft rots developed and were coloured regularly in alternate zones of light and dark brown. The tissue beneath the cork layers near the infection site was suberized. Suberization involved about 6 cells in a radial direction from the wound and decreased gradually in the deeper tissues. The thickness of the walls of the suberized parenchymatous cells near the cork layers reached 3 to 4μ. Disintegration of some of the cells



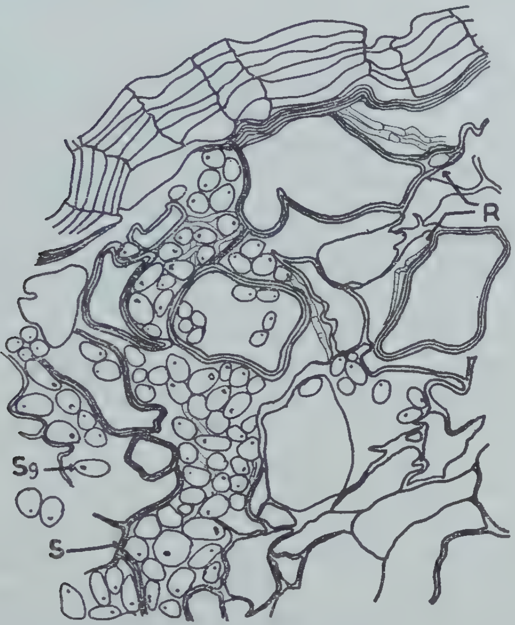
TEXT-FIG. 8. Section in potato tuber infected with *Rhizoctonia solani* then *Fusarium solani*, and kept over saturated solution of calcium chloride, showing fungi in the vascular tissue and in parenchyma surrounding it (× 125).

R: *Rhizoctonia solani*
F: *Fusarium solani*
S: suberized cell walls
N: normal cell walls

surrounding the wound formed by the cork borer was observed. In this place starch grains accumulated. Concurrently, the other cells which remained became separated from each other. The walls of these were found to be considerably thickened, attaining 9μ thickness (Text-fig. 9). At the base of the wound, 3 or 4 layers of cells were partially suberized, but were invaded by *Rhizoctonia*. Thickened haustoria of *Fusarium* were seen in the advanced regions of invasion. In advance of the fungi, meristematic cells were formed. The fungi, after penetrating a small distance, advanced more rapidly in the vascular tissue than in the parenchymatous tissue, the former becoming surrounded by suberized tissue.

At 100 per cent. R.H. the soft rot appeared, as in treatments 3 and 4, in light and dark brown zones. Moreover, in most specimens the rot spread in a horizontal direction directly beneath the cork layers. The two fungi were found externally, growing vigorously from the inoculum to the outside, where 'black scurfs' were formed. Intense invasion of the parenchymatous cells by *Rhizoctonia* was observed, and in some cases the cell walls disappeared and big sclerotia were formed in place of the disintegrated cells. Suberization of tissues occurred as in treatments 3 and 4.

In treatment 2, although the expected humidity was lower than that of treatment 3, the observed humidity was higher. Despite the high observed humidity of treatment 2, its rot was drier than in treatment 3. This can be explained as follows. The initially low relative humidity in treatment 2 leads to the formation of a dry rot, which allows a high rate of evaporation, thus enabling the water from the tuber to replace that absorbed by the solution. In treatments 3, 4, 5, where the water-absorbing capacity of the solutions used was low, a soft rot results, hindering evaporation from within the tubers. Thus the relative humidities are nearer to the expected ones.



TEXT-FIG. 9. Section of infected potato tuber kept over 30 per cent. H_2SO_4, showing disintegration of cells and thickening of the walls of the other cells (× 125).

R: *Rhizoctonia solani* S: thickened cell wall
Sg: starch grain

DISCUSSION

In potato tubers the relations following an infection by *Fusarium* in a site previously infected with *Rhizoctonia* showed a 'mutualism' (Clarke, 1954); the two species each derived benefit from the association because they were able to make persistent advance in this substratum when associated, whereas they were unable to do so when grown individually. This example also shows 'synergism' (Holman, 1928), because the two fungi working together bring about a change which neither could produce alone. For the following reasons this example cannot be considered as a case of primary and secondary parasitism: firstly, despite the successful rotting when *Rhizoctonia* was the first to be inoculated, it was subsequently *Fusarium* which was the first to advance intercellularly; secondly, the phenomenon was not simply *Rhizoctonia* helping *Fusarium* to advance: *Fusarium* also helped *Rhizoctonia*. Thus this association is a mutual relationship, which can be called 'synergistic mutualism'.

Different types of growth were manifested by the two fungi in individual as compared with joint infections. *Rhizoctonia* alone grows abundantly as sclerotial cells inside the host cells, passing from cell to cell. Ramsey (1917) reported that the potato cells collapse some distance in advance of *Rhizoctonia* hyphae, and that actual penetration of cells was rarely found. However, in the present investigation, when a *Rhizoctonia* infection was followed by *Fusarium*, the *Rhizoctonia* hyphae advanced easily to the inner tissues of the tuber, forming restricted patches of sclerotia and yielding evidence of both inter- and intracellular hyphae. *Fusarium*, as an individual growth, was present mainly in the intercellular spaces, with haustorial penetration of cells. When infection occurred after *Rhizoctonia*, fewer haustoria were observed, and the peculiar phenomenon of conidial formation inside the cells of hypertrophied regions of the tubers seen as superficial 'pimples' was noted (Elarosi, 1956).

Longer *Fusarium* haustorium-like structures were formed in mature tubers than in young ones. This may have been due to the presence of a greater concentration of available nutrients in the young tubers than in the mature ones. A similar case occurred in the Peronosporales (Fraymouth, 1956), where it was observed that the amount of nourishment available is the primary factor determining the final form of haustoria. In healthy leaves the haustoria are relatively simple and few in number, but in the pith or cortex of a stem, where there is likely to be less available food, they are usually more elaborately developed. Fraymouth also showed that the host tissue by checking hyphal growth stimulates the formation of haustoria. An analogous situation may be the cause of the more frequent formation of haustoria by *Fusarium* when it infects alone, than when it follows *Rhizoctonia*.

Pimple-like structures similar to the ones appearing here in the double infection, have elsewhere been reported to occur in potato tubers as a response to fungal infection (Kunkel, 1915; Millard and Burr, 1923).

It seems to the writer that cultural and physiological studies on the two fungi and their association might help in elucidating the factor(s) interfering in this association. These studies will be dealt with in further papers.

ACKNOWLEDGEMENTS

The writer wishes to acknowledge the invaluable criticism, and the continuous guidance, of Professor C. W. Wardlaw, during the progress of this work. Sincere thanks are also due to Dr. D. Park for his valuable help. The preparation of the photographs was carried out by Mr. E. Ashby and Mr. G. Barker, to whom the writer expresses his thanks.

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EXPLANATION OF PLATES

PLATE XXII

Fig. 1. Potato cells infected with *Rhizoctonia solani* (× 500).

Fig. 2. Intercellular hypha and haustorium-like structures of *Fusarium solani* (× 1,000).

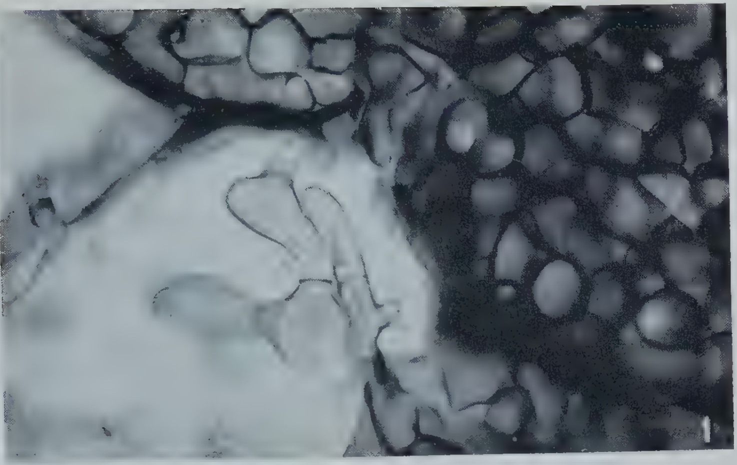
Fig. 3. Section in a dark core formed inside rotted tissue of potato tuber infected with *Rhizoctonia solani* followed by another infection with *Fusarium solani* (× 150).

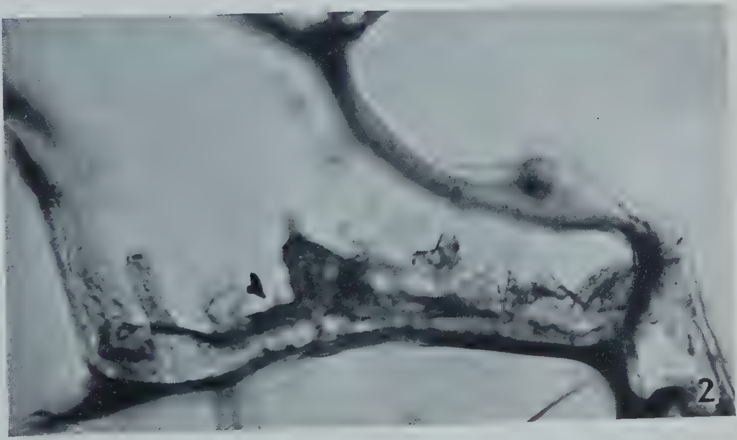
PLATE XXIII

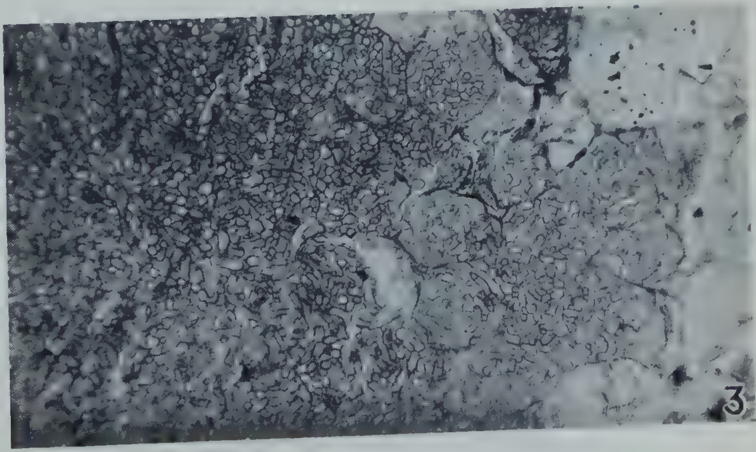
Fig. 4. Potato tubers infected with *Rhizoctonia solani* then *Fusarium solani*, showing pimple-like outgrowths (normal size).

Fig. 5. Tubers in Fig. 4 after cutting into halves (normal size).

Fig. 6. Tubers infected with *Fusarium solani* then *Rhizoctonia solani* (normal size).







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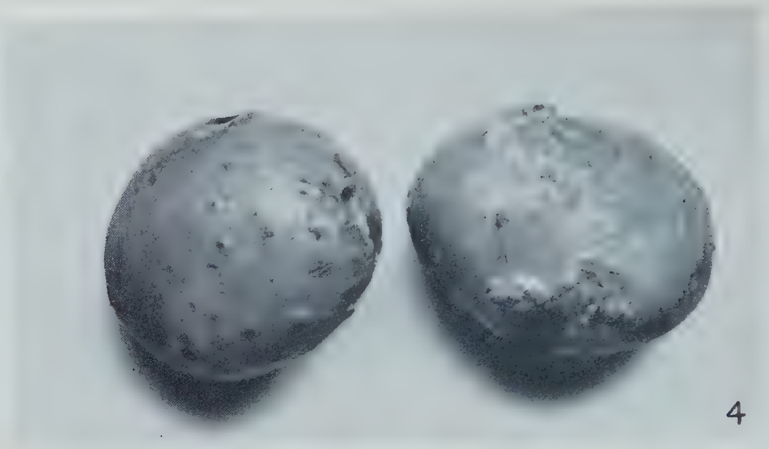

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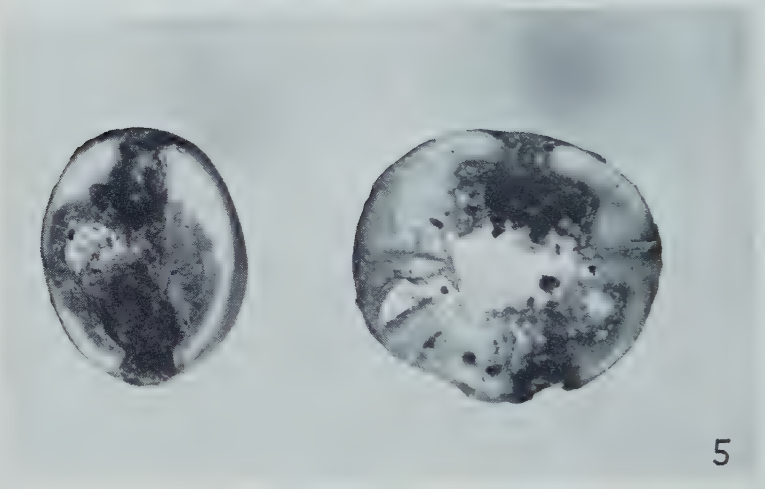

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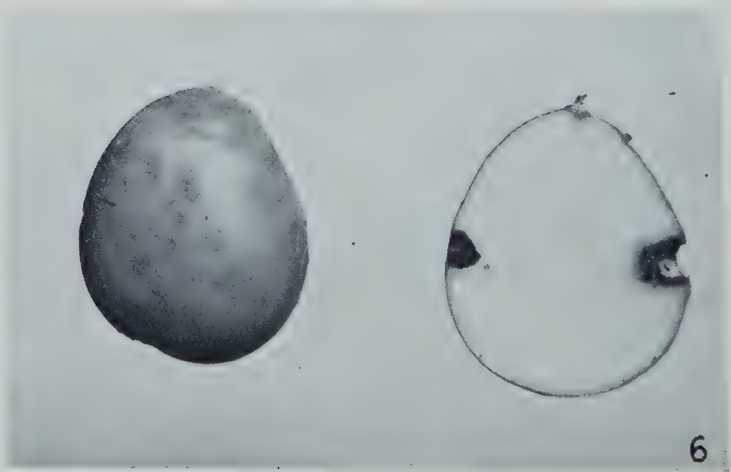

6

H. ELAROSI

Fungal Associations

II. Cultural Studies on *Rhizoctonia solani* Kühn, *Fusarium solani* Snyder and Hansen, and Other Fungi, and Their Interactions

BY

HUSSEIN ELAROSI

(*Department of Cryptogamic Botany, University of Manchester*)

With Plates XXIV and XXV and three Figures in the Text

ABSTRACT

Several types of interaction between *Rhizoctonia solani*, *Fusarium solani*, and *Phoma foveata* were found when these fungi were grown on potato-dextrose agar. After being used by *Rhizoctonia* a potato mash medium gave better growth of *Rhizoctonia* and *Fusarium* than it did when the medium was initially used by *Fusarium*; and this was so whether the reaction of the spent medium was readjusted or not. It is suggested that potato mash medium used by *Fusarium* contains a thermostable factor(s) affecting the subsequent growth of *Rhizoctonia* or *Fusarium*.

The range of pH values suitable for *Rhizoctonia* growth was narrower than that for *Fusarium*, optimum values being approximately 5·9 for the former and 7·8 for the latter. In mixed cultures of the two fungi on potato-dextrose agar adjusted to different pH values, the fungus for which the reaction of the medium was more suitable usually became visually predominant after some time.

A study of various carbon sources showed that poor growth of *Rhizoctonia* was obtained when pectin was used as the sole source of carbon. On a pectin-agar medium, the rate of growth of a *Rhizoctonia* colony increased on the sector which lay towards an adjacent *Fusarium* colony; also, after the two fungi came in contact, there was more rapid growth of *Rhizoctonia* round the *Fusarium* colony than elsewhere. On a synthetic liquid medium with pectin as the carbon source better *Rhizoctonia* growth was obtained when *Fusarium*-spent medium was added to it than when *Rhizoctonia*-spent medium was added.

Rhizoctonia showed partial deficiencies in thiamine, biotin, and inositol. Both the extract of *Fusarium* mycelium, grown on vitamin-free medium, and the *Fusarium*-spent medium, stimulated the growth of *Rhizoctonia* on vitamin-free medium.

INTRODUCTION

AS the histological study of having potato tubers infected by *Rhizoctonia solani* followed by *Fusarium solani* (Elarosi, 1957) did not give a clear picture of the relationships of the two fungi during their synergistic action, cultural studies of the fungi, individually and in association, were made in an effort to obtain fuller information on this aspect. In some experiments, other fungi were introduced to help in the interpretation of the data.

HISTORICAL

Numerous investigations have been made on microbial associations in culture media. In studies of the reactions of one organism to another in culture media, Porter (1924) showed that when the mycelium of one fungus approached that of another, one of five types of reactions may occur. Arrillaga

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(1935) recognized four of Porter's types, and introduced three additional ones. The effects of micro-organisms, their extracts, or their metabolites, on the sporulation of others have been investigated by Heald and Pool (1908), Wilson (1927), Asthana and Hawker (1936), and Grossbard (1955).

Ecologists have demonstrated that the growth of an organism may greatly modify the substratum and that the changes induced may make it more suitable than before for the growth of certain other organisms; the latter may in turn modify the substratum still further. The succession of higher plants is gradual, but that of micro-organisms is usually rapid (Cook, 1924). The rate of decomposition of alfalfa by *Trichoderma* sp., for example, is considerably modified by the presence of various fungi and bacteria (Waksman and Hutchings, 1937). Rege (1927), using rice-straw as a cellulosic material, demonstrated that combinations of *Acremoniella* sp., *Aspergillus* sp., and *Coprinus* sp., in two's gave better decomposition than each fungus individually; but all three together led to the most rapid decomposition. In most cases of succession, the first micro-organism decomposes some initial compound, thereby providing a suitable substrate for the second organism, the latter being unable to utilize the initial compound. Alternatively, the first organism forms a complex compound from simpler ones, the second organism requiring this complex material, but being unable to form it from the original substratum. Already in 1899 Ward had shown the importance of micro-organismal succession in completing certain processes of decomposition

MATERIALS AND METHODS

Fungi were kept as stock cultures on slopes of potato-dextrose agar. Cultures were renewed every 2 to 3 months by transferring inocula to new medium, the cultures being closely examined for variability.

Potato mash, potato decoction, potato dextrose, and synthetic solutions based on Brown's (1925) medium were used in different experiments, the latter being used in nutrition experiments. In the carbon-source experiments, the carbon compounds were added at the rate of 4 gm. carbon per litre. In nitrogen-source experiments, the nitrogen compound added contained equivalent amounts of nitrogen to that in 2 gm. asparagin per litre; these being added to a basic medium containing 20 gm. glucose per litre. In studies of vitamin deficiencies Analar substances were used. Micro-elements were added at the rate per litre of

0·7 mgm. $Fe_2(SO_4)_3$;
0·9 mgm. $ZnSO_4.7H_2O$;
0·4 mgm. $MnSO_4.4H_2O$.

EXPERIMENTAL RESULTS

(*a*) *Effect of one fungus on the growth of another*

Petri-dishes of potato-dextrose agar were each inoculated with two fungi, 4·5 cm. apart, along a diameter, *Rhizoctonia solani*, *Fusarium solani*, and *Phoma foveata* being used in different combinations. When the reactions occurring here were compared with those described by Porter (1924) and Arrillaga (1935), only one of Porter's type reactions was found, the others not being recognized by these authors. The three types observed by the writer can be characterized as follows:

Type I. Slight inhibition: equivalent to Porter's type C. Both organisms show some reduction in growth, but they approach each other until almost in contact, when growth ceases. This type was found when the inocula were either of *Rhizoctonia* or of *Fusarium*, and less clearly when *Fusarium* and *Phoma* approached each other.

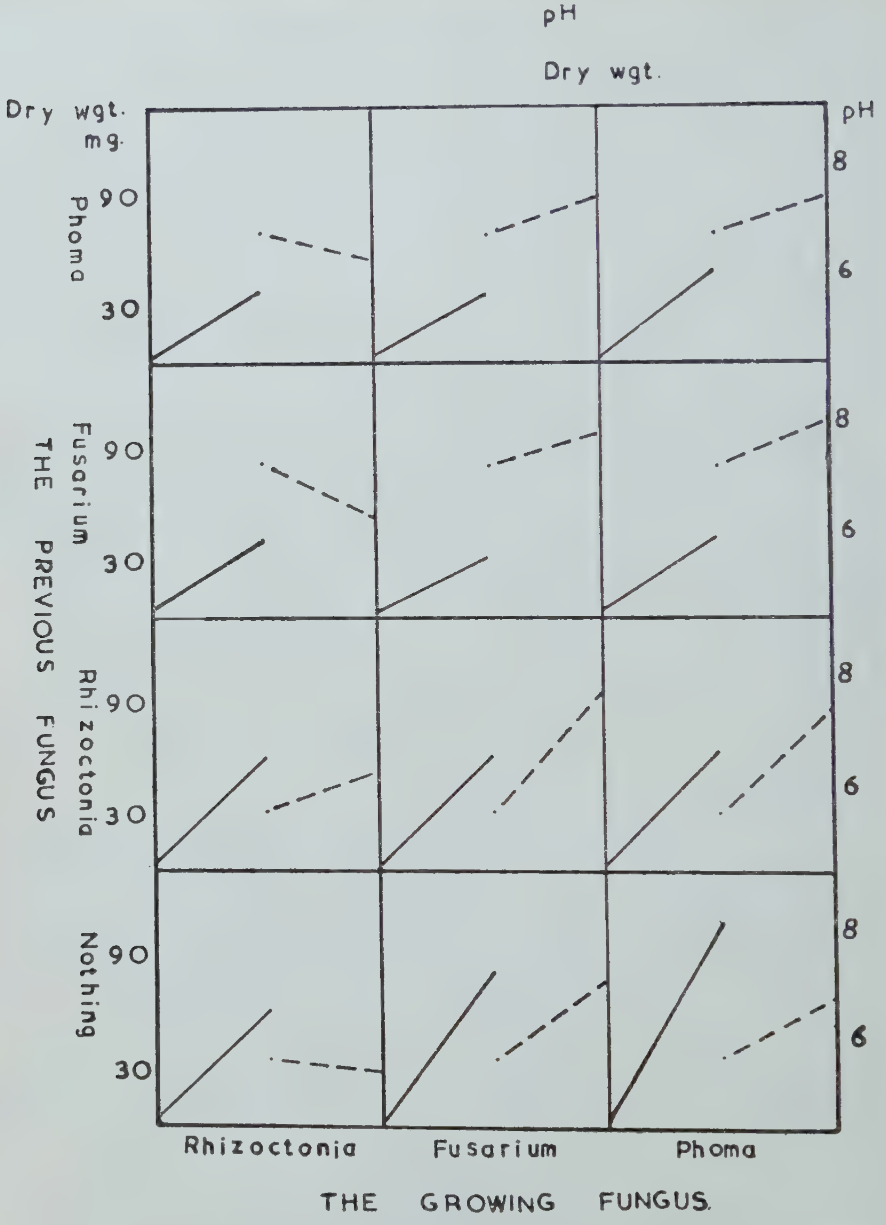
Type II. Slight intermingling: the two converging organisms show a diminishing rate of growth, but grow over each other for a short distance, after which their growth ceases. This type was observed when *Phoma* approached another growth of *Phoma* or of *Rhizoctonia*.

Type III. The two colonies show no sign of inhibition until they come into contact; one of them is inhibited but the other grows over the inhibited colony, with striking morphological changes over the latter. This was demonstrated by *Fusarium* and *Rhizoctonia*, the latter growing over the former and forming abundant sclerotia over and round it. Sclerotia were also formed on the pure *Rhizoctonia* colony but only near the inoculum on the side towards *Fusarium* (Pl. I, Fig. 1).

In the writer's experiments, *Rhizoctonia* showed slight inhibition towards another colony of *Rhizoctonia*, and *Fusarium* showed the same towards another growth of *Fusarium*; yet this inhibition was not found between *Fusarium* and *Rhizoctonia* colonies. Moreover, as indicated above, *Fusarium* stimulated sclerotial formation by *Rhizoctonia*.

To clarify and amplify the foregoing results, the relationships of medium initially used by one of the fungi in supporting the growth of one of the others were studied. Cultures were grown in 250 ml. conical flasks containing 35 ml. potato mash medium of pH 5·8. After 10 days' incubation at room temperature (16–22° C.), the mycelium was filtered off, and its dry weight, and the pH value of the used medium, determined. These media were resterilized and used for growing another fungus. Text-fig. 1 shows the dry weights reached by each fungus on different media during 10 days' incubation, and the corresponding changes in the pH values of the media. The curves show the initial dry weights and those obtained at the end of the incubation period. The greatest growth of each fungus was obtained in new medium, this being followed by the growth in medium previously used by *Rhizoctonia*. Poor growth was obtained after *Fusarium* and after *Phoma*. After the first growth of *Fusarium* and of *Phoma*, the pH values of the media were raised, while after a first growth of *Rhizoctonia* the pH values decreased slightly. In all treatments, the growth on the used media raised the pH values, except in the case of *Rhizoctonia* when used after *Fusarium* or *Phoma*, where a marked decrease of pH values resulted.

It seems that a positive correlation exists between the hydrogen-ion concentration of the media after the initial culture (pH was 5·6 after *Rhizoctonia*,



TEXT-FIG. 1. Fungal growth and pH changes of media previously used by fungal growth.

6·7 after *Phoma*, and 7·2 after *Fusarium*), and the amount of growth in the second culture. Consequently, it became important to ascertain if the good growths obtained after *Rhizoctonia* were due to the low pH value after its growth, to the lower consumption of nutrients by this fungus as compared with *Fusarium* and *Phoma*, or to some other factors provided by *Rhizoctonia*.

Rhizoctonia and *Fusarium* were used to study the effect on subsequent growths of adjusting spent media to the original pH. After 10 days' growth on potato mash, mycelia were removed and the media were readjusted to the original pH value (5·5). The second treatments were carried out as shown in Table I. In treatments 3 and 4 the adjusted old media were mixed with new ones in equal amounts. The amount of medium used was the same in each of the five treatments (25 ml.). The results show that, despite the uniformity in pH, better growth was still obtained after *Rhizoctonia* than after *Fusarium*. When new medium was added to the used one, the differences between the growths obtained were not significant. It was inferred that potato mash medium used by *Fusarium* contained a thermo-stable substance(s) which affected both *Rhizoctonia* and a second growth of *Fusarium*, the effect being greater at higher concentrations.

TABLE I

Effect of Previous Growth on Fungal Activity, after Adjustment of Spent Medium to Original Value

	R. solani		*F. solani*	
Medium used	pH	Dry wt. mgm.	pH	Dry wt. mgm.
1. *R. solani*-used medium . .	5·4	290	8·8	160
2. *F. solani*-used medium . .	5·1	170	8·7	103
3. ½ *R. solani*+½ new medium .	5·1	313	8·8	280
4. ½ *F. solani*+½ new medium .	5·1	307	8·8	243
5. New medium	5·3	317	8·7	393
L.S.D.	—	61	—	42

TABLE II

Effect of Several Previous Growths on Fungal Activity, after Adjustment of Spent Medium to Original Value

	R. solani		*F. solani*	
The present growth→ The previous growth ↓ . .	pH	Dry wt. mgm.	pH	Dry wt. mgm.
1. Nothing then *F. solani* . .	5·8	417	7·1	472
2. *F. solani* then *R. solani* . .	6·3	207	7·9	332
3. *R. solani* then *F. solani* . .	5·7	330	7·8	520
L.S.D.	—	48	—	45

It was of interest to know whether the substance(s) formed by *Fusarium* could still be formed after the second crop of *Rhizoctonia*, or whether it became ineffective. Accordingly, 40 ml. were used as the initial amount of potato mash per 250 ml. flask. In each flask three successive 10-day cultures were taken except in the first treatment (Table II). Media were autoclaved and the reaction readjusted to the original pH value after each crop. In a comparison between the growth of *Rhizoctonia* or *Fusarium* after two successive crops, one of *Rhizoctonia* and the other of *Fusarium*, it was found that significantly better growths of both fungi were obtained after *Rhizoctonia* followed by *Fusarium* than with the reverse arrangement. In comparing the growths after one crop of *Fusarium* with growths after two successive crops of the two fungi it was found that growth after one crop is better than after two crops, except in the case of *Fusarium* in media used by *Rhizoctonia* followed by *Fusarium*.

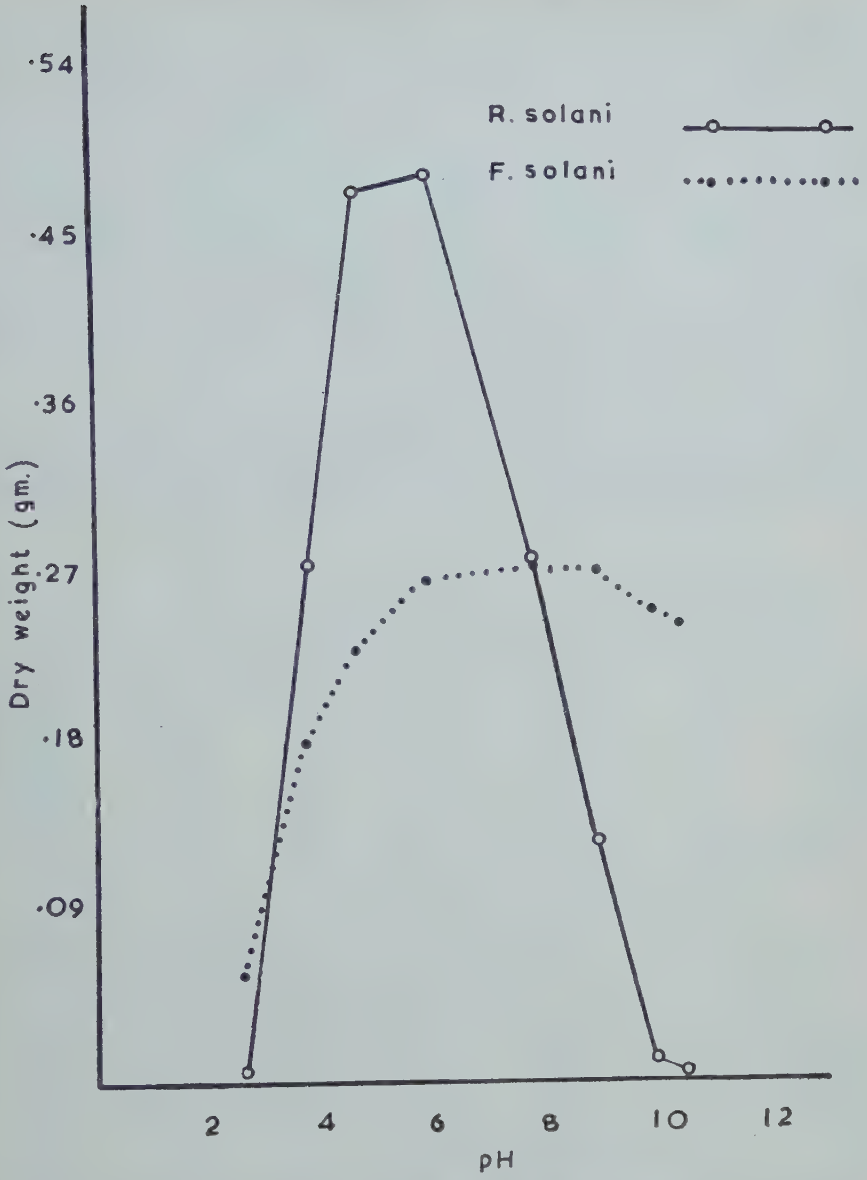
This experiment shows that the factor formed by *Fusarium* which affects subsequent growths of *Rhizoctonia* and *Fusarium* was not affected by a subsequent crop of *Rhizoctonia*. However, when *Fusarium* grew in a medium previously used by *Rhizoctonia*, the formation of the factor was less intense, especially on a third crop of *Fusarium*. This may be due to the smaller amount of growth of *Fusarium* when grown in *Rhizoctonia*-used medium than when grown in new medium.

(*b*) *Effects of pH on growth of* Rhizoctonia solani *and* Fusarium solani

The results illustrated in Text-fig. 1 and set out in Tables I and II show that the changes in the pH of media used by *Rhizoctonia* were very slight when the initial pH was round 5·5, but that the decrease was greater when the pH value was initially higher. However, in all cultures of *Fusarium* on media of pH about 5·5, a rise in pH values occurred. Thus it was thought that pH values after growth of one fungus may have a stimulatory or inhibitory effect on the next fungus in the succession. The pH ranges for both fungi were, therefore, determined.

Potato-dextrose broth, adjusted to a range of pH values from 2·6 to 10·5, was used. Cultures were incubated for 10 days at room temperatures (17–22° C.), after which dry weights were obtained. The pH values of media had a great effect on the dry weight of mycelia (Text-fig. 2), especially with *Rhizoctonia*, where the pH range suitable for growth was narrower than that for *Fusarium*. Almost no growth of *Rhizoctonia* was obtained at pH values 2·6 and 9·9, while poor growth only was obtained at 2·6 with *Fusarium*. Optimum pH values were approximately pH 5·9 for *Rhizoctonia* and 7·8 for *Fusarium*. On this basis, then, the pH of the potato mash after 10 days' growth of *Rhizoctonia* (Text-fig. 1) is suitable for further growth of *Rhizoctonia*, and can support good growth of *Fusarium*. The change in pH after the 10 days' growth of *Fusarium*, however, is suitable for *Fusarium*, but less suitable for *Rhizoctonia*. It can therefore be inferred that, as far as pH is concerned, growth of *Rhizoctonia* maintains the suitability of the medium for its further growth, and may also postpone the trend towards the staling point of *Fusarium*.

The selective effects of pH on fungal growth in mixed cultures were studied, potato-dextrose agar medium, adjusted to a range of pH values, being used. Inocula were taken from submarginal regions of a mixed culture previously grown on plain agar. After 10 days' growth, *Rhizoctonia* was seen to be the dominant organism at pH values of 5·3 and 6·5, while *Fusarium* was dominant at values of 3·7, 8·8, and 10·0. At pH 7·9 *Fusarium* was dominant for the first 6 days, after which *Rhizoctonia* overcame the influence of



TEXT-FIG. 2. Effect of pH of medium on growth of *Rhizoctonia solani* and *Fusarium solani*.

Fusarium and became dominant. *Fusarium* continued growth, parasitizing *Rhizoctonia* by coiling round the aerial hyphae in straight or wavy hyphae, in the same manner as does *Trichoderma lignorum* when parasitizing *Rhizoctonia solani* (Weindling, 1932). It has previously been reported that *Rhizoctonia solani* can be attacked by different species of fungi including *Fusarium* (Weindling, 1934). At pH 5·3 and 6·5, *Rhizoctonia* sclerotia were formed round and over the restricted growth of *Fusarium* which had appeared in the first few days of growth.

The effects of pH on mixed cultures are partially explicable by those of the pH range experiment. Thus *Rhizoctonia* was dominant at pH values round the optimum for its growth, i.e. 5·3 and 6·5. *Fusarium* was dominant at 8·8 and 10·0, largely because these values support good growth of *Fusarium* and do not suit *Rhizoctonia* growth. A pH value 3·7, although supporting moderate growth of both fungi separately, allowed *Fusarium* to become dominant in the mixed culture. At pH 7·9, which is near the optimum for *Fusarium* and which supports moderate growth of *Rhizoctonia*, *Fusarium* was the dominant, but only for 6 days. Here the high rate of growth of *Rhizoctonia* seems to be the cause of its ultimate dominance.

(*c*) *Effects of the source of carbon on fungal growth*

Some nutritional requirements of *Rhizoctonia solani* and *Fusarium solani* have been investigated, mainly in an attempt to analyse the synergistic relation between the two fungi in rotting potato tubers (Elarosi, 1957).

TABLE III

The Effect of the Carbon Source on the Linear Growth of Rhizoctonia solani

	Diameter (mm.) after periods of time (days)				
Carbon source	1	2	3	4	5
Glucose	19·5	32·0	68·5	88·0	118·5
Maltose	14·0	26·5	45·5	68·0	110·0
Sucrose	14·0	24·5	44·5	67·5	106·5
Starch	12·0	28·5	61·5	84·0	126·0
Pectin	12·0	28·5	40·0	50·0	74·5
Cellulose	12·0	28·0	48·0	77·5	114·0
No carbon source	12·0	24·5	40·0	62·5	85·4

TABLE IV

The Effect of the Carbon Source on the Linear Growth of Fusarium solani

	Diameter (mm.) after periods of time (days)				
Carbon source	2	4	6	8	10
Glucose	18·0	41·5	69·5	98·0	124·0
Maltose	18·0	42·0	72·0	100·0	127·5
Sucrose	18·0	40·0	68·0	98·0	124·0
Starch	19·0	42·0	72·0	101·5	128·0
Pectin	20·0	42·5	70·5	100·5	128·5
Cellulose	18·5	45·0	72·5	102·0	128·0
No carbon source	20·0	46·0	72·5	101·5	128·0

Rhizoctonia and *Fusarium* were grown separately, at 18–21° C., on synthetic agar media with different carbon sources selected on the basis of their presence in host tissue. Tables III and IV show the radial growth of the two fungi at different successive periods. The best growth of *Rhizoctonia* was obtained with starch and glucose, and least with pectin. Moreover, the rate of growth was lower when pectin was used in the control, where carbon source was lacking. Nevertheless, on the 10th day, big and mature sclerotia were present in pectin medium in an area of 2 cm. diameter round the inoculum, while a few small white sclerotia were scattered in an area of 3 cm. diameter round the inoculum in the control. When starch or glucose was used, brown sclerotia were formed throughout in the dish in the course of 10 days. Sucrose gave the same result, but the sclerotia did not become brown until the 10th day. With maltose, brown sclerotia were formed, densely crowding over each other, but restricted to an area of 1 to 2 cm. round the inoculum. Sclerotial formation in cellulose medium was similar to that in the control, but the sclerotia were bigger.

The differences in the rate of *Fusarium* growth on different carbon sources were small (Table IV). Although growth-rates were not affected by the carbon sources used, there were notable differences in the densities of growth, and in the colours of the media and mycelia. These may reflect the utilization of carbon. Thus growth was feeble and transparent in the control and on cellulose, whereas it was dense and coloured on the other media. On the 10th day, a white growth was obtained when starch was used, 'primrose yellow' with glucose, maltose, and sucrose, and 'cartridge buff' with pectin. Cellulose did not support the growth of *Fusarium*, the high linear rate of growth in this case and in the control being an expression of starvation growth.

TABLE V

Effect of Carbon Sources on Fungal Growth

		R. solani		*F. solani*	
Carbon source	Initial pH	pH after growth	Dry wt. mg.	pH after growth	Dry wt. mg.
Glucose . . .	7·36	6·04	200	7·80	153
Starch . . .	8·47	6·46	187	7·92	133
Pectin . . .	4·40	4·11	63	7·27	123
Control . . .	8·37	8·26	37	8·54	37
L.S.D. . . .	—	—	23	—	26

Dry weights of two fungi grown in liquid media containing glucose, starch, and pectin as carbon sources were obtained (Table V). No significant differences were noted between the amounts of growth with glucose and with starch media when either *Fusarium* or *Rhizoctonia* was used. Pectin gave good growth of *Fusarium*. This was significantly different from its growth on glucose, but not from that on starch. *Rhizoctonia* growth on pectin was very poor, though better than in the control. This poor growth cannot be attributed to the initial pH of the medium, since with pectin this was more suitable for *Rhizoctonia* growth than with other carbon sources (Text-fig. 2). With *Fusarium*, on the other hand, the smaller amount of growth on pectin than on glucose may be attributable to the low initial pH value.

(*d*) *The relation between* Rhizoctonia solani *and* Fusarium solani *when pectin is the carbon source*

The foregoing experiments showed that feeble growth of *Rhizoctonia* was obtained with pectin as the carbon source, whereas the growth of *Fusarium* was relatively good on the same medium. Accordingly, it seemed worth while to study the relation between these two fungi in media containing the carbohydrates. The two fungi were grown from inocula 4·5 cm. apart in Petri-dishes, using glucose, starch, pectin, cellulose, and a control lacking a carbon source. Slight inhibition between the two fungi equivalent to Type I was observed in all the treatments, except in the case of pectin. Here a new type of reaction was observed which may be called Type IV. In this type, the rate of growth of one of the organisms increases as it approaches the other, but only in the sector adjacent to the second organism; the latter grows at a constant rate in all directions till the two colonies meet. In the experiment under consideration, the *Rhizoctonia* colony showed an increase in growth towards the *Fusarium* colony from the second day onward (Table VI). After the two fungi had come into contact, there was more rapid growth of *Rhizoctonia* round the *Fusarium* than elsewhere (Pl. I, Fig. 2).

TABLE VI

The Effect of Fusarium solani *Growth on the Linear Growth of* Rhizoctonia solani *in Pectin Medium*

	½ diameter (mm.) after periods of time (days)		
	1	2	3
Growth towards *Fusarium* . .	6·67	14·67	23·33
Growth perpendicular to *Fusarium* .	6·67	13·67	22·00

Thus, in all, three kinds of reaction have been obtained between *Rhizoctonia* and *Fusarium*, i.e. Type I in synthetic media without pectin, Type III in potato-dextrose agar, and Type IV in synthetic medium with pectin. Stimulation effects occurred either with pectin as carbon source or when potato-dextrose was used, the stimulation being more pronounced with the former. As pectin is known to be one of the constituents of potato tubers, it is reasonable to assume that it, or degradation products, is present in potato-dextrose agar. An explanation of the stimulation, on this basis, requires that pectin, or some of its degradation products, is connected with the stimulatory effects in both media, and that the effect becomes more obvious when the pectin is more concentrated.

TABLE VII

The Effect of Growth of Rhizoctonia solani *and* Fusarium solani *in Pectin Medium on Further Growth of these Fungi*

The previous fungus	The growing fungus	Initial pH	pH after 1st crop	pH after 2nd crop	Dry wt. mg.
Rhizoctonia	*Rhizoctonia*	4·40	4·11	6·57	50
Fusarium	,,	4·40	7·27	8·77	23
Rhizoctonia	*Fusarium*	4·40	4·11	8·19	67
Fusarium	,,	4·40	7·27	8·23	30

To test the last conclusion, the succession of *Rhizoctonia* and *Fusarium* on liquid synthetic medium with pectin as carbon source was studied. After 10 days' growth of individual fungi, the used media were sterilized by Seitz filtering and again inoculated (Table VII). The dry weights show that better growth of both fungi were obtained after *Rhizoctonia* than after *Fusarium*. These unexpected results, which disagree superficially with those just cited, and which do not indicate any stimulatory effect of *Fusarium* towards *Rhizoctonia*, may be explained by one of the following assumptions: (*a*) that the raising of the pH value after the first crop of *Fusarium* had a retarding effect on the second crop; (*b*) that the initial growth of *Fusarium* exhausted all or nearly all the pectin, leaving the medium with an insufficient carbon source; (*c*) that the Seitz filter did not allow the stimulating factor to pass through.

TABLE VIII

The Subsequent Effect on Rhizoctonia solani *of Growth of* Rhizoctonia solani *and* Fusarium solani *in Pectin Medium after Adjusting pH Values of Used Media*

Media used for second growth	pH after 2nd growth	Dry wt. mg.
1. Medium used by *Rhizoctonia*	6·97	23
2. ,, ,, ,, *Fusarium*	6·98	20
3. ,, ,, ,, *Rhizoctonia* mixed with new medium	6·33	67
4. ,, ,, ,, *Fusarium* mixed with new medium	7·23	123
5. New medium	7·56	107
L.S.D.	—	29

The succession was again studied, but with adjustment of the spent media to the original pH value. Also, possible effects of nutrient exhaustion were reduced in one series of treatments by mixing used and unused media in the ratio of 1:4 before growing the second crop. The results (Table VIII) show that significant differences between *Rhizoctonia* growth on media previously used by *Rhizoctonia*, on the one hand, and *Fusarium* on the other hand, did not exist when the pH values of media were adjusted. But a significant difference was obtained with the mixture of used and unused media, better growth being obtained after *Fusarium* than after *Rhizoctonia*. Also, the good growth obtained on new medium plus *Fusarium*-used medium was better than that on new medium alone, although the difference was not statistically significant. It is now clear, therefore, that the lowering of hydrogen-ion concentration after *Fusarium* growth and medium exhaustion after the first crop were involved in the failure to demonstrate the stimulatory effect after *Fusarium* in the previous experiment.

As pectin is an integral part of the cell-wall, pieces of potato tubers were used to demonstrate the effect of the previous combination and others on the utilization of these pieces, and the effect of such pieces on fungal growth.

The two fungi were inoculated as far apart as possible on plain agar plates.

Six-mm. diameter potato cylinders, taken from the centre of King Edward tubers, were cut to 2 to 3 mm. thickness, surface-sterilized with calcium hypochlorite (10 gm. per 140 ml. distilled water) for 20 min. and washed several times with sterilized distilled water (Mead, 1933). When the two fungi came in contact on the plain agar surface, the sterilized potato discs were placed over the cultures in two lines at right angles to each other, one along the line between the two inocula and the other along the line of contact between the two fungi. After 4 days the discs on the line of contact were being attacked vigorously, whereas those on either the *Rhizoctonia* or the *Fusarium* growths were attacked only to a much smaller degree.

When various other fungi were used instead of *Fusarium solani*, the following results were obtained:

1. With *Fusarium solani*, *F. roseum* (Dis.), and *F. roseum* (Ros.),[1] it was observed that *Rhizoctonia* grew vigorously with sclerotial formation round and on the potato pieces placed on the line of contact between the two fungi, but less growth and no sclerotial formation appeared round the other pieces, or between the two fungi in places devoid of potato discs (Pl. II, Fig. 3 *a*, *b*, *c*). Moreover, the three *Fusaria* gave more intensive colours on and round the potato at the line of contact than where discs were placed on the *Fusarium* growth alone. The deterioration of potato pieces was more advanced in the line of contact, where they were softened and macerated, than on other parts, where the fungi grew separately, and where, after the 4 days, they were still somewhat hard.

2. With *Phoma foveata*, potato discs placed on the pure *Phoma* colony were most affected. A small amount of infection occurred in the line of contact of the colonies. The least infection was over the pure *Rhizoctonia* growth (Pl. II, Fig. 3*d*).

3. With *Cylindrocarpon radicicola* Wollenweber and *Verticillium* sp. the potato discs were but slightly attacked in all positions (Pl. II, Fig. 3*e*).

(*e*) *Interaction between* Rhizoctonia solani *and* Fusarium solani *on different nitrogen sources.*

Three compounds were chosen as representatives of the three main nitrogen sources, namely, asparagin as an organic source, ammonium chloride as ammonium nitrogen, and sodium nitrate as nitrate nitrogen. The two fungi were inoculated at opposite sides of the agar plates. Slight inhibition (Type I) was observed with asparagin and sodium nitrate, and also in the control. With ammonium chloride, a type of reaction, not mentioned by Porter (1924) or Arrillaga (1935), was observed: it consisted in a strong inhibition from one side only and will here be called Type V. *Rhizoctonia* ceased growth at the 8th day, while the colony was still about 10 mm. distant from *Fusarium*. Thereafter *Fusarium* continued to grow very slowly until it came quite close to the *Rhizoctonia* colony, when a slight inhibition (Type I) was observed. Microscopic examination of the marginal hyphae of *Fusarium* showed normal morphology. *Rhizoctonia*, on the contrary, showed a departure from the normal on the side towards *Fusarium*, the tips of the main hyphae bending away from *Fusarium* (Text-fig. 3 A and B), while the side branches grew out at a greater angle than usual, sometimes exceeding a right angle; and tertiary branches were mostly on the side away from the *Fusarium*. The walls of the terminal cells tended to be corrugated (Text-fig. 3 C and D).

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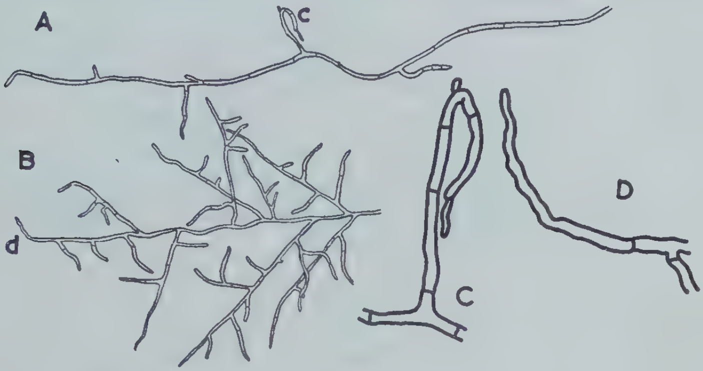
[1] The *Fusarium* spp. were classified after Snyder and Hansen (1940), the two *F. roseum* cultures being distinguished according to the Sections of Wollenweber and Reinking (1935), i.e. Discolor and Roseum.



TEXT-FIG. 3. The inhibited marginal growth of *Rhizoctonia solani* on the side of the colony near *Fusarium solani* growth, with ammonium chloride as nitrogen source (× 50 for A and B, × 250 for C and D).

(*f*) *Vitamin deficiencies*

The vitamins used for this study, and their concentrations, were as follows: thiamine 100 μg. per litre; biotin 5 μg. per litre; inositol 5 μg. per litre; pyridoxine 100 μg. per litre. The fungi were grown in 15 ml. medium in 100 ml. conical flasks for 10 days, and dry weights determined (Table IX).

TABLE IX

Effect of Vitamin Sources on Fungal Dry Weight

Treatment	Dry weight (mg.) *Rhizoctonia*	Dry weight (mg.) *Fusarium*
1. Basic medium (vitamin-free)	30	193
2. ,, ,, +thiamine	140	183
3. ,, ,, +biotin	160	177
4. ,, ,, +inositol	140	170
5. ,, ,, +pyridoxine	87	177
6. ,, ,, +thiamine+biotin	183	183
7. ,, ,, + ,, + ,, +inositol+pyridoxine	173	180
L.S.D.	59	—

Because of the high value of the least significant difference between dry weights of *Rhizoctonia solani* at different treatments, no significantly clear differences were found between them. Certain differences were, however, visually evident. Treatments 2, 3, 4, 6, and 7 yielded better growth than that of the control. Treatment 5 gave less growth than the other vitamins, and it was not significantly different from the control. With thiamine, biotin, and inositol, whether used separately or in combination, the improvement in growth as compared with the control was nearly the same. This finding that any one of these three vitamins could replace either of the other two is unusual in vitamin deficiency investigations. It may be explained by assuming that the deficiency in these vitamins is merely a partial one, and that any one of them may stimulate the formation of the others, but further research would be necessary to substantiate this assumption.

TABLE X

Effect of Vitamin-free Medium Used by Fusarium solani *on Subsequent Growth of* Rhizoctonia solani

Treatment	Dry wt. mg.
1. Vitamin-free medium	50
2. ,, ,, +Seitz-filtered mycelium	133
3. ,, ,, +autoclaved mycelium	167
4. ,, ,, +Seitz-filtered medium	147
5. ,, ,, +autoclaved medium	97
L.S.D.	57

Since an analysis of variance of the *Fusarium solani* dry weights showed that the treatment variance was smaller than the error variance, it can be stated that any apparent difference between treatments was due solely to error in random sampling (Paterson, 1939). Hence *Fusarium* may be considered self-sufficient, i.e. autotrophic, in respect of the vitamins tested (thiamine, biotin, inositol, and pyridoxine).

Spent, initially vitamin-free, medium, after *Fusarium* culture, was examined for its ability to provide *Rhizoctonia* with the vitamins in which it is deficient. 15 ml. of vitamin-free medium in 100 ml. conical flasks was inoculated with *Fusarium*. After 10 days' incubation the mycelium was separated from the medium, washed with distilled water, then dried under vacuum at room temperature, after which it was ground up and extracted with 15 ml. of distilled water, i.e. the original volume of the medium in which the mycelium was grown. The filtered spent medium was also made up to 15 ml. Both solutions were divided into two parts, one of each being Seitz-filtered and the other autoclaved. In preparing the test media, 2 ml. hyphal extract, or 2 ml. used medium, were added to 13 ml. sterilized, vitamin-free medium in 100 ml. conical flasks. The flasks were then inoculated with *Rhizoctonia* and incubated for 10 days. The results recorded in Table X show that both the hyphal extract and the *Fusarium* filtrate stimulated the growth of *Rhizoctonia*.

DISCUSSION AND CONCLUSIONS

(*a*) *Growth relations of* Rhizoctonia solani *and* Fusarium solani

That the relation between *Rhizoctonia solani* and *Fusarium solani* when grown together is not a simple one, and that it varies in character according to the substratum, is shown by the experimental records presented. In fact, the relationship observed in some instances would be classified under 'symbiosis' and in others under 'antagonism', as defined by Clarke (1954), whose terminology is adopted here.

1. When infection of potato tubers by *Fusarium* takes place in sites previously infected by *Rhizoctonia*, the two fungi stand in a relationship which may be indicated as *synergistic mutualism* (Elarosi, 1957).

2. In a synthetic culture medium with pectin as carbon source, the rate of growth of *Rhizoctonia* was lower than with other carbon sources; this rate was increased in the vicinity of a *Fusarium* colony, the *Fusarium* itself being unaffected. This effect of *Fusarium* on *Rhizoctonia* is indicative of *commensalism*, one species being benefited while the other suffers no adverse effect.

3. When ammonium chloride was used as the nitrogen source in a synthetic medium, a zone of inhibition remained between the colonies of the two fungi, due to the cessation of *Rhizoctonia* growth towards *Fusarium*. This sort of antagonism, which is attributed to harmful secretions from *Fusarium*, is included in the category of *antibiosis*.

4. *Competition* between the two species is seen in cultures in which the two fungi have grown from a mixed inoculum, the fungi competing for space, food, &c. At different pH values, only one of the fungi was demonstrably present at the periphery of the colony at the end of the experiment.

5. *Parasitism* by *Fusarium* on *Rhizoctonia* was observed in mixed cultures grown on potato-dextrose agar of pH 7·9, and exemplifies *exploitation*.

The writer's observations support the view, based on other investigations, that environmental conditions are important in determining the kind of association in which parasitic fungi may coexist. Broadfoot (1933), in a study of the antagonistic and compatible relationships of 66 cultures of bacteria and fungi towards *Ophiobolus graminis*, found that many of the micro-organisms which controlled to a marked degree the virulence of *O. graminis* towards wheat seedlings in soil, were not antagonistic to the pathogen on potato-dextrose agar or on Molisch's salt-peptone agar. On the other hand, many of the organisms which had little or no effect on the virulence of *O. graminis* in soil were decidedly antagonistic on potato-dextrose agar; and many that were compatible with the pathogen on potato-dextrose agar were antagonistic on Molisch's salt-peptone agar. Broadfoot explained these findings on the assumption that micro-organisms, each with its complement of enzymes, produce substances which differ in kind and amount according to the substratum. The interrelationships of certain fungi growing together on the same substratum may also be affected by other environmental factors, e.g. Savastano and Fawcett (1929) found that certain fungal mixtures which depressed or accelerated the rotting of citrus fruits did so only within a specifiable temperature range.

(*b*) *The synergistic mutualism of* Rhizoctonia solani *and* Fusarium solani *in the rotting of potato tubers*

The association between the two fungi was primarily studied in order to investigate factors relating to this phenomenon.

1. *Buffer action. Rhizoctonia* may alter the pH of the substratum to a value suitable for the growth of *Fusarium.* When *Rhizoctonia* was grown on potato mash used by *Fusarium*, the pH value of the medium, which was previously raised by the growth of *Fusarium*, was reduced to a figure approximating to the original one. Hence it is thought that the presence of *Rhizoctonia* along with *Fusarium* in potato tissue might tend to maintain the pH of the tissue close to its original value for a longer period than with *Fusarium* alone and reduce the latter's staling effect. Boyle (1924) and Pratt (1924) showed that the staling effect of *Fusarium* spp. can be counteracted in part by restoring the pH value.

2. *Vitamin deficiencies.* The possibility that *Fusarium* may have a stimulating effect on *Rhizoctonia* by providing it with vitamins in which it is deficient was examined and it was shown that the feeble growth of *Rhizoctonia* on a vitamin-free medium is improved both by the addition of a small quantity of vitamin-free medium on which *Fusarium* has been grown and by a water extract of the *Fusarium* mycelium from that medium. *Sclerotinia rolfsii*, which showed poor growth on a synthetic medium lacking thiamine, grew well in the presence of other micro-organisms (Morten and Stroube, 1955).

3. *Pectic enzymes.* The observation that there is a stimulatory effect of *Rhizoctonia* on *Fusarium* when pectin is the sole carbon source, and that a pectin medium used by *Fusarium* stimulates *Rhizoctonia* in pectin medium, has drawn the author's attention to the possibility that pectic enzymes may be important in this situation. Further investigations on this aspect will be considered in a further paper.

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EXPLANATION OF PLATES

PLATE XXIV

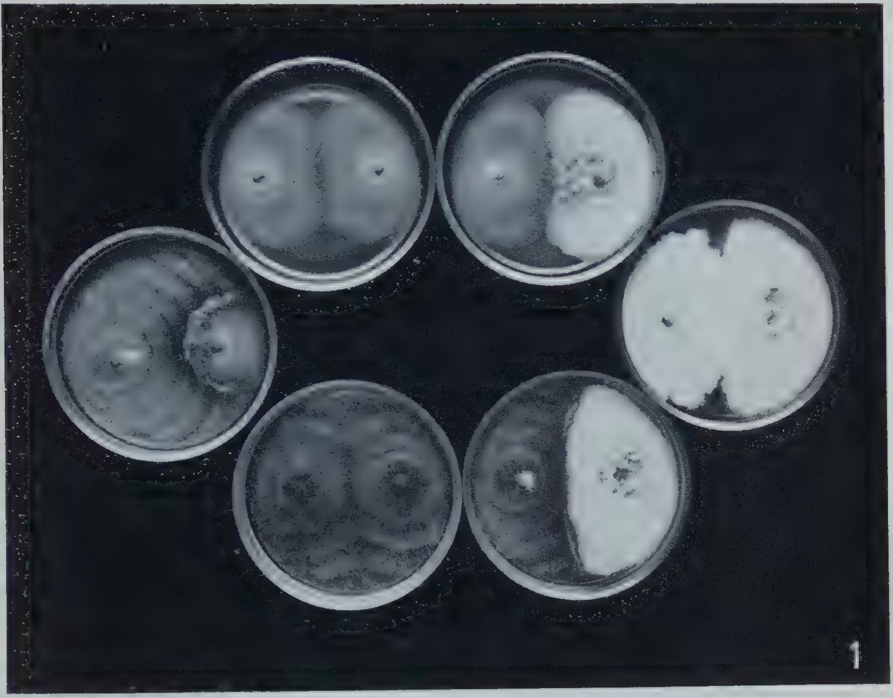
Fig. 1. Reactions of fungi towards each other in 10-day-old cultures, fungi arranged as follows:

(*Fusarium—Fusarium*) (*Fusarium—Phoma*)
(*Rhizoctonia—Fusarium*) (*Phoma—Phoma*)
(*Rhizoctonia—Rhizoctonia*) (*Rhizoctonia—Phoma*)

Fig. 2. Stimulation of *Rhizoctonia solani* growth towards *Fusarium solani*, left: the pectin as carbon source; right: starch as carbon source.

PLATE XXV

Fig. 3. Effect of mixed cultures with *Rhizoctonia solani* on infection of potato discs after 4 days; upper fungus is *Rhizoctonia*, the lower ones are, (*a*) *Fusarium solani*, (*b*) *F. roseum* (Dis.), (*c*) *F. roseum* (Ros.), (*d*) *Phoma foveata*, (*e*) *Verticillium* sp.



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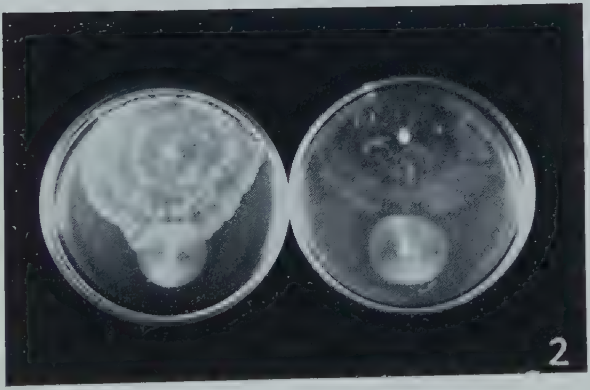

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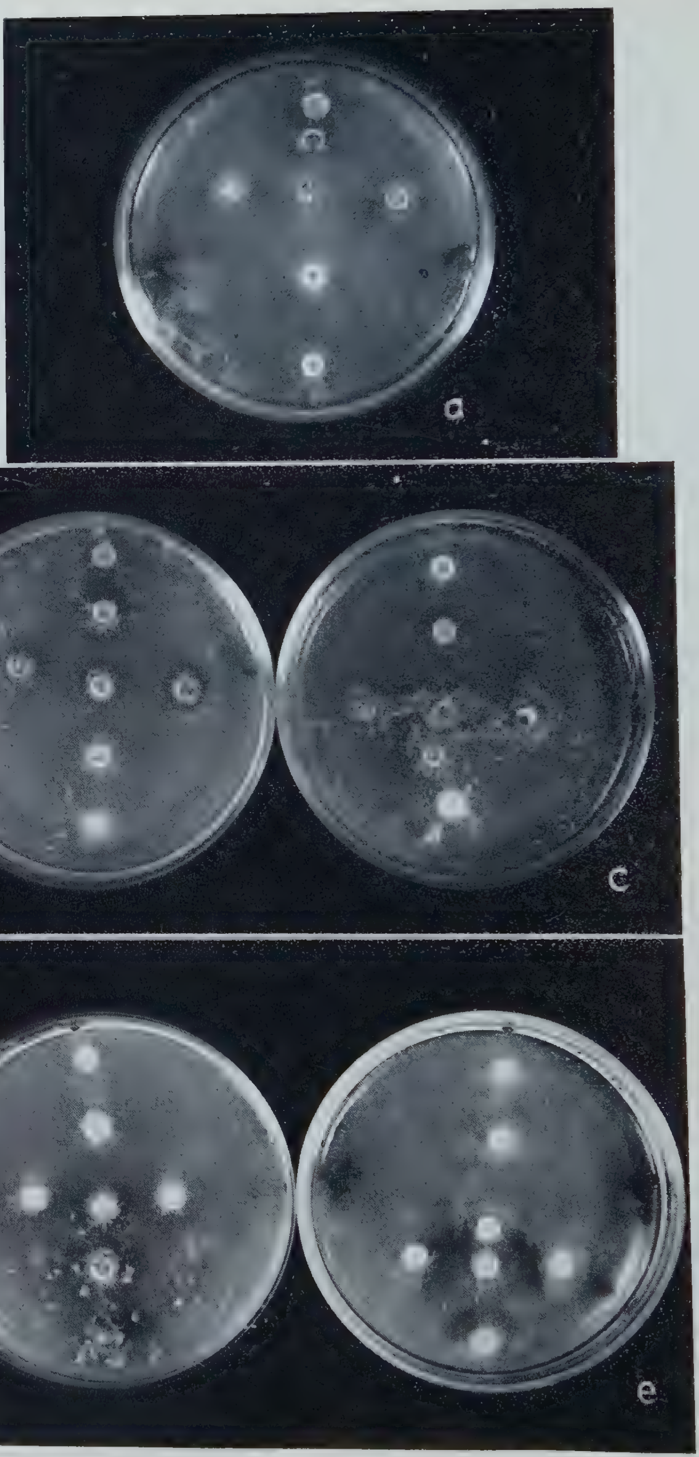

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The Function of Trace Metals in the Nitrogen Metabolism of Plants

BY

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ABSTRACT

In recent years trace metals have been found to be associated with flavoproteins, a number of which are key enzymes in the nitrogen metabolism of plants. Thus Mo is a constituent of nitrate reductase which mediates the reduction of nitrate to nitrite in some bacteria, fungi, and higher plants. During the enzymic reduction of nitrate a one-electron change is involved, $Mo^{6+} \rightleftharpoons Mo^{5+}$. Nitrite and hyponitrite reductases both require Fe and Cu and flavin for maximum activity and are inhibited by uncoupling reagents so that a phosphorylation takes place at these two reductive steps. Hydroxylamine reductase, a flavoprotein that is Mn-dependent, but, as with nitrate reductase, is unaffected by uncoupling reagents. An aromatic route may also exist in plants for the reduction of organo-nitro compounds, but it is more likely that the aliphatic route is the primary reductive pathway.

Mo, V, W, and Fe may all be involved in the process of N_2 fixation in free living micro-organisms and in the root nodule bacteria. The suggestion that the enzyme hydrogenase is coupled to N_2 fixation is not borne out by the results of valency experiments with Mo and V which show that an active hydrogenase does not necessarily result in a vigorous fixation process.

Although Mo has been shown to be a constituent of hydrogenase from *Clostridium pasteurianum*, experiments in this laboratory show that a deficiency of iron only reduced the enzyme in this organism and in *Azotobacter*. Both Mo and Fe, however, are essential for N_2 fixation. In some strains of *Azotobacter*, V or W can partially replace Mo for N_2 fixation.

INTRODUCTION

IRON, copper, zinc, manganese, molybdenum, and boron are known to be essential for the growth of green plants. More recently chlorine was shown to be required by a variety of higher plants (Stout *et al.*, 1954), sodium by the blue-green alga *Anabaena cylindrica* (Allen and Arnon, 1955), and vanadium by *Scenedesmus obliquus* (Arnon *et al.*, 1955).

Two methods used to establish the essentiality of a mineral nutrient for growth are (*a*) its omission from the culture solution resulting in a reduction in growth which can be remedied only by returning the nutrient to the plant in a readily available form, (*b*) determining a function for the metal in the metabolism of the organism. In practice the first approach is often a prerequisite to the second.

The first method often involves the use of rigorous purification techniques to remove the metal from the basal culture solutions and it has been used to good effect with relatively simple media including salts and sugars. With complex media, however, further specialized procedures must be used to

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avoid loss of the organic constituents (Nicholas, 1952). Method (*b*) is direct but it is difficult to establish with certainty the sites and mode of action of metals in enzyme systems. This method, however, has been effective in recent years in establishing specific functions for metals known to be required for growth and may well play an important part in the discovery of other essential metals.

The criteria for the essentiality of an element for plant growth as defined by Arnon (1950) has been a useful guide in plant nutrition studies. As a result of recent work, his concept that an element is essential for growth only when it cannot be replaced by another needs revision. Thus the chlorine requirement of green plants can be replaced by bromine and, to a lesser extent, by iodine but at much higher levels than for chlorine. It is, however, reasonable to assume that chlorine is the most readily available halide when plants grow in their natural habitats and that only under exceptional conditions would Cl or I_2 substitute for it. Chlorine is therefore an essential element. In certain *Azotobacter* strains, V or even W can partially replace Mo in the fixation of N_2, thus all three nutrients function in metabolism. It is therefore better to redefine an essential element as one that functions in normal metabolism, then it may be termed a 'metabolism nutrient'.

NITRATE ASSIMILATION

Aliphatic route

The assimilation of nitrate nitrogen by plants involves the reduction of nitrate to ammonia resulting in a reduction change of the nitrogen atom from +5 to −3, involving a net change of 8 electrons.

The importance of molybdenum in the reduction sequence was established when it was found that nitrate accumulated and free amino-acids were depleted in molybdenum-deficient tissue of fungi (Nicholas, 1950) and green plants (Mulder, 1948; Hewitt and Jones, 1952). These effects, however, are not specific for Mo as a deficiency of Mn (Leeper, 1941) may result in a similar phenomenon. It was also established that the Mo requirements of fungi and higher plants were substantially reduced when ammonium ions were substituted for nitrate as the sole source of nitrogen.

(i) *Nitrate reductase*

The attempts to demonstrate the presence of nitrate reductase enzyme in cell-free extracts of plants have been reviewed in detail by Nightingale (1937, 1948), Bürstrom (1939), Virtanen and Rautenen (1952), and more recently by Nason (1956) and Evans (1956).

An enzyme has been isolated from fungi and higher plants (Nason and Evans, 1953), which catalyses the reduction of nitrate to nitrite. This enzyme was shown to be a flavoprotein with flavin adenine dinucleotide (FAD) as the native flavin and reduced di- or triphosphopyridine nucleotide (DPNH or TPNH) as the electron donors. In *Neurospora* and *Aspergillus* (Evans and Nason, 1953; Nicholas and Nason, 1954), TPNH is the specific donor, whereas in higher plants either nucleotide can function. Stoicheiometric experiments showed that for each mole of TPNH oxidized one mole of nitrite was formed:

$$NO_3^- + TPNH + H^+ \rightarrow NO_2^- + TPN + H_2O$$

Metal constituent. The enzyme was shown to be sensitive to metal inhibitors including cyanide, azide, and 8-hydroxyquinoline. Using the purification methods developed at Long Ashton (Nicholas, 1952), it was possible to produce individual deficiencies of the trace metals in *Neurospora*, including those for Mo and Cu. The results are shown in Table I.

TABLE I

Effect of individual Nutrient Deficiencies in Neurospora *on Nitrate Reductase in Cell-Free Extracts of the Felts*

(Wild Type 146)

Units of enzyme activity mμM NO_2 formed per mg. of protein as per cent. of control values

Culture solutions	Control	Omit Ca	Omit N*	Omit Mg*	Omit Fe	Omit Cu	Omit Zn	Omit Mn	Omit Mo	Omit Biotin
Per cent. growth .	100	16	8	20	0·7	40	28	30	29	15
Nitrate reductase .	100	146	12	90	190	292	116	113	10	80

* N and Mg were supplied at one-thirtieth and one-hundredth, respectively, of the level in the controls.

A deficiency of Mo only has resulted in a significant reduction in nitrate reductase. The enzyme was increased when Cu, Zn, or Fe were deficient. The low enzyme activity in the omit N felts is probably caused by a restricted synthesis of protein which would affect most enzymes.

The data in Table II indicate that the enzyme formation is dependent on Mo when nitrate, nitrite, or ammonium nitrate are the sources of nitrogen. The enzyme is not formed when ammonia is the sole source of nitrogen and under these conditions the Mo requirement is considerably reduced but not eliminated. Arnon *et al.* (1955) have recently shown that in *Scenedesmus obliquus* the Mo requirement is abolished when either ammonia or urea is substituted for nitrate as a nitrogen source. This result is, however, contrary to that obtained with fungi (Mulder, 1948, and Nicholas, 1950) or with higher plants (Hewitt and Bolle-Jones, 1952).

The evidence thus far showed that Mo nutrition affected nitrate reductase, but whether the trace element was a constituent of the enzyme or whether it exerted its effect indirectly was yet to be determined (Nicholas, Nason, and McElroy, 1953). It was shown that a range of other enzymes requiring reduced pyridine nucleotides for their activity were unchanged in Mo-deficient felts (excepting TPNH-dependent glutamic dehydrogenase, which was reduced by the deficiency). Further, the riboflavin content of normal felts were comparable with those deficient in Mo.

The results of the following experiments showed Mo to be a constituent of nitrate reductase. An increase in the specific activity of the enzyme in

TABLE II

Effect of Molybdenum Deficiency and Nitrogen Source on Nitrate Reductase in Cell-Free Extracts of N. crassa *and* A. niger

Units of enzyme activity mμM NO_2 formed/10 min./mg. of protein in the extract

Fungus	Nitrogen source	Treatment	Per cent. growth	Nitrate reductase
Neurospora (Wild type 146 microconidial)	Nitrate	+Mo	100	21
		− ,,	29	5
	Nitrate and ammonia	+ ,,	100	17
		− ,,	30	10
	Nitrite	+ ,,	100	24
		− ,,	40	1
	Ammonia	+ ,,	100	2
		− ,,	70	0
Neurospora (Wild type 5297a macronidial)	Nitrate	+ ,,	100	11
		− ,,	32	1
	Nitrate and ammonia	+ ,,	100	19
		− ,,	24	1
	Nitrite	+ ,,	100	34
		− ,,	45	11
	Ammonia	+ ,,	100	3
		− ,,	65	1
Aspergillus (M strain) . .	Nitrate	+ ,,	100	39
		− ,,	5	2
	Nitrate and ammonia	+ ,,	100	42
		− ,,	75	20
	Ammonia	+ ,,	100	0
		− ,,	80	0

various protein fractions of *E. coli*, *Neurospora*, and soybean leaves (Nicholas and Nason, 1954, &c.) was paralleled by an increase in their Mo contents. The other trace metals examined were not correlated with the enzymatic activity. This effect was later confirmed by Evans (1955), who showed that ^{99}Mo accumulated in active nitrate reductase preparations from soybean.

Molybdenum was removed from a purified enzyme by dialysis against glutathione and cyanide, and incubation of the dialysed and washed preparation with either molybdenum trioxide or sodium molybdate restored its activity to about 80 per cent. of that of the undialysed enzyme. No other trace metal could substitute for Mo in the reactivation process, as shown in Table III.

Evans (1956), using a similar dialysis technique with enzyme from soybean leaves, confirmed that a dialysed enzyme was reactivated by Mo and he also showed a small stimulation by the addition of Fe salts. The 'Fe effect' may well have been caused by the presence of Mo as a contaminant in the Fe compounds used. Pure iron salts do not appear to reactivate the enzyme.

Mechanism of action. The role of Mo in the reduction sequence is now established to be as follows:

$$\begin{matrix}\text{TPNH}\\ \text{or}\\ \text{DPNH}\end{matrix} \rightarrow \begin{matrix}\text{FAD}\\ \text{or}\\ \text{FMN}\end{matrix} \rightarrow \text{Mo} \rightarrow NO_3^- \rightarrow NO_2^-$$

TABLE III

Effect of Metals on Reactivation of Cyanide-Dialysed Nitrate Reductase

Units of enzyme activity per 0·05 ml. of enzyme

Experiment No.	Treatment 1: Undialysed enzyme	Treatment 2: Dialysed for 6 hrs. against 0·1 M phosphate + 10^{-3} M glutathione*	Treatment 3: Same as Treatment 2 + 10^{-3} M cyanide	Treatment 4: Same as Treatment 3, then redialysed for 6 hrs. as in Treatment 2
I	18	14	4	4
II	15	11	3	4

Metals added after Treatment 4

Experiment No.	MoO_3	Na_2MoO_4	$FeCl_3$	$FeSO_4$	$ZnSO_4$	$MnSO_4$	$NiCl_2$
I	13	14	5	6	4	3	4
II	12	11	4	4	3	3	2
	$AgCl$	$NaWO_4$	$Na_2Cr_2O_7$	Na_3VO_4	$VO_2(NO_3)_2$	$CoSO_4$	$Na_2B_2O_4$
I	4	4	5	3	4	3	4
II	3	3	4	3	4	3	4

* Reagents freed from molybdenum.

1 μg. of each metal added to 0·05 ml. of enzyme and incubated for 10 min. at 25° before assay.

It was found that the reduction of FAD or FMN by DPNH or TPNH was mediated equally well either by the purified enzyme or by the Mo-free enzyme. Only the normal enzyme was capable of transferring electrons from reduced flavin to nitrate, but the addition of Mo to the Mo-free enzyme restored its capacity to do this. It was then shown that sodium molybdate reduced with sodium dithionite could act as an effective electron donor for the enzymatic reduction of nitrate (Table IV).

The results established that the element was undergoing electron change during nitrate reductase action. The valence states of molybdenum, viz. Mo^{6+}, Mo^{5+}, and Mo^{3+}, were separated by means of paper chromatography and it was shown that the most reduced valence state in the hydrosulphite-reduced molybdate is Mo^{5+} (Nicholas and Stevens, 1955). The effect of Mo^{5+}, prepared free from the other valence states, as an electron donor for the enzymatic reduction of nitrate is shown in Table V.

Mo^{5+} was as effective as TPNH as an electron donor for the enzymic reduction of nitrate. Mo^{6+}, however, was without effect. In the absence of the enzyme or in the presence of the boiled enzyme Mo^{5+} did not reduce nitrate.

Mo^{3+} was prepared by reducing acidified sodium molybdate with Zn dust. The excess Zn and residual Mo^{5+} were removed from solution on a cellulose column. Mo^{3+} thus prepared reduced nitrate non-enzymically whereas Mo^{5+} or Mo^{6+} did not. It is unlikely, however, that Mo^{3+} is important in plant metabolism as it is readily oxidized to Mo^{5+} in the presence of Mo^{6+}, Fe^{3+}, or Cu^{2+}, which are invariably present in plant tissues. Mo^{4+} is only stable in strong ethanol and it dismutes readily in the presence of a trace of water

TABLE IV

Dithionite-treated Molybdate as Electron Donor for Enzymatic Nitrate Reduction

Millimicromoles of nitrite formed per 0·5 ml. of reaction mixture*

Experiment No.	TPNH, enzyme, FMN, KNO_3	Reduced Mo,† enzyme, KNO_3	Mo, enzyme, KNO_3	Reduced blank,‡ enzyme, KNO_3	Reduced Mo,† KNO_3	Reduced Mo,† boiled enzyme, KNO_3
1	24	19	7	3	1	4
2	30	25	9	8	2	6
3	20	15	2	2	1	2
4	23	20	4	2	2	3

* 0·30 ml. of enzyme containing approximately 500 μg. of protein, 0·24 ml. of TPNH (2 μM per ml.), 0·24 ml. of FMN (0·09 μM per ml.), 0·6 ml. of 0·1 M KNO_3, and 0·5 ml. of 10^{-3} M $Na_2MoO_4 . 2H_2O$; made up to 3·0 ml. with 0·1 M pyrophosphate buffer, pH 7·0. Reactions started by tipping the enzyme and nitrate into the other components of the reaction mixtures in evacuated modified Thunberg tubes. After 10 mins.' incubation at room temperature the mixtures were assayed for nitrite with sulphanilamide and N-(1-naphthyl)-ethylenediamine reagents.

† Reduced molybdate was prepared in the Thunberg tubes by adding approximately 2 mg. of $Na_2S_2O_4$ to 0·5 ml. of 10^{-3} M $Na_2MoO_4 . 2H_2O$ contained in the pyrophosphate buffer. H_2 gas was then bubbled through the solution for 10 mins. followed by evacuation, incubation, and testing as indicated above.

‡ $Na_2S_2O_4$ and H_2 treatment as for 'reduced Mo' but without molybdate.

TABLE V

Mo^{5+} as an Electron Donor for the Enzymatic Reduction of Nitrate

Millimicromoles (mμM) nitrite formed in the reaction mixture

Experiment No.	TPNH, enzyme, FMN, KNO_3	Mo^{5+}, enzyme, KNO_3	Mo^{5+}, KNO_3	Mo^{5+}, boiled enzyme, KNO_3	Mo^{6+}, enzyme, KNO_3
1	35·5	36	0	2	0
2	24	22	0	1	0

0·5 ml. of enzyme containing approximately 400 μg. of protein, 0·20 ml. TPNH (2 μM/ml.), 0·20 ml. FMN (0·09 μM per ml.), 0·6 ml. 0·1 M KNO_3, and 50 μg. Mo^{5+} or Mo^{6+} contained in 0·01 ml., checked for purity by paper chromatography. The reaction mixtures were made to 2 ml. with 0·1 M phosphate buffer pH 7·5. After 10 min. at room temperature the mixtures were assayed for nitrite by using sulphanilamide and N-(1-naphthyl)ethylenediamine reagents.

to give Mo^{3+} and Mo^{5+}; the former would then be readily oxidized to Mo^{5+}.

Thus it appears that during the enzymic reduction of nitrate, Mo undergoes a one-electron change so that two Mo^{5+} atoms would be required to accept the two electrons from reduced flavin.

The purified enzyme (over 80-fold) was shown recently to require phosphate for maximal activity (Nicholas and Scawin, 1956), and is thus similar to other metallo-flavoproteins (Mahler, 1955). The phosphate is required for the molybdenum to function in the enzyme as an acceptor or donor of electrons, and is not necessary for the first stage in the sequence, i.e. TPNH → flavin. Although phosphomolybdate alone is usually unstable at an alkaline pH where the enzyme is active, the apoenzyme may well stabilize this complex so as to allow it to undergo a valency change. Arsenate, tellurate, selenate, and silicate can replace phosphate presumably because they also form complexes with molybdates and have similar atomic radii to phosphate, being within the range 2·4 to 2·8 Å. Uncoupling reagents have no effect on the enzyme so that it is unlikely that a phosphorylation is involved in this step (Nicholas and Scawin, 1956).

Distribution of the enzyme. Nitrate reductase occurs in bacteria, fungi, and higher plants (Evans and Nason, 1953, &c.; Nicholas and Nason, 1954, &c.; Evans, 1955). In soybean inoculated with pure cultures of *Rhizobium japonicum* grown without combined nitrogen, an active nitrate reductase was found in the bacteria (Evans, 1955). He has suggested that chemisorbed N_2, or an oxidized product, may induce the adaptive formation of nitrate reductase in the nodules. Although this is an attractive hypothesis, it is known that traces of nitrate only are required for the adaptive formation of the enzyme and in the author's experience it is difficult to eliminate NO_3 completely from salts used in the culture solutions. The enzyme is not present in plants given ammonium salts as the sole source of nitrogen but it will adapt to the addition of nitrate. Plants deficient in molybdenum contain little enzyme but injection of the micro-nutrient into the leaves results in its reconstitution within a few hours.

Sato and Egami (1956) have shown that nitrate reduction in *E. coli* is also mediated by an enzyme containing a cytochrome-b type pigment which is inhibited by 2-heptyl 4-hydroxyquinoline N-oxide indicating the presence of haem iron. In the double beam spectrophotometer he observed an oxidation of cytochrome-b during the enzymic reduction of nitrate. It is feasible that both the iron and the molybdoflavoprotein systems exist for nitrate reduction in organisms, although at the present time evidence for the latter system is more complete.

(ii) *Nitrite reductase*

Nitrite reductase was first demonstrated in cell-free extracts of *Bacillus pyocyaneus* by Yamagata (1939), and in *B. pumilis* (Taniguchi, Sato, and Egami, 1956) showed that nitrite was reduced to ammonia by reduced methylene blue. It is unfortunate that much of the Japanese work is based on the use of artificial carriers because the native constituents of their systems have not been identified.

A nitrite reductase has been found in *Neurospora* and in soybean leaves which utilizes 3 moles of TPNH or DPNH for each mole of nitrite reduced to ammonia (Nason and Evans, 1953). The enzyme is a flavoprotein which is inhibited by metal-binding agents. The enzyme from soybean leaves was stimulated by Mn (Nason, Abraham, and Averbach, 1954), but this effect, which was based on the oxidation of DPNH in the extracts in the presence of NO_2^-, may well occur at any of the enzymic steps between NO_2 disappearance and NH_3 production. Recent work in this laboratory (Medina and

Nicholas, 1957*b*, *c*) has shown that nitrite is enzymically reduced to hyponitrite in *Neurospora*. With the use of an improved assay method for the enzyme (Medina and Nicholas, 1957*a*), it has been found that the enzyme is reduced in felts deficient in Cu or Fe. Uncoupling reagents, metal chelating agents, and *p*-chloromercuribenzoate inhibit the enzyme.

(iii) *Hyponitrite reductase*

This enzyme, first identified in cell-free extracts of *Neurospora*, is a flavoprotein requiring DPNH as the donor for the reduction of hyponitrite to hydroxylamine (Medina and Nicholas, 1957*b*). The enzyme is inhibited by quinoline, and cyanide, by uncoupling reagents, and by -SH inhibitors. As with nitrite reductase, the enzyme is markedly reduced in felts deficient in Cu or Fe. It is likely that nitrite and hyponitrite reductases are distinct enzymes because the reduction of product of nitrite reductase (hyponitrite) inhibits the enzyme and a Zn-deficiency which depressed nitrite reductase was without effect on hyponitrite reductase (Medina and Nicholas (in press)). The results of the enzyme assay in cell-free extracts of *Neurospora* are shown in Table VI.

TABLE VI

Hyponitrite reductase in Neurospora

Experiment No.	(mμM NH_3 produced in 20 min./ml. enzyme)			Specific Activity of complete system mμM NH_3/20 min. /mg. protein
	Complete system	Omit DPNH	Omit enzyme	
1	288	48	0	34·7
2	237	49	0	28·5
3	411	51	0	28·3

Complete System: Outer cell of Conway unit contained 0·15 ml. 0·2 M pyrophosphate (pH 7·5); 0·1 ml. 4×10^{-3} *M* $Na_2N_2O_2$; 0·05 ml. boiled pig heart; 0·2 ml. enzyme (8·3 mg./ml.); 0·1 ml. 10^{-2} *M* DPNH.

Inner cell 1·5 ml. 0·01 *N* HCl.

All reagents (except enzyme) were boiled before assay to eliminate ammonia.

Incubated for 20 min., then 1 ml. saturated K_2CO_3 added to outer cell followed by further incubation for 1½ hr. 0·4 ml. of inner solution tested for NH_3 using the phenate-hypochlorite method.

(iv) *Hydroxylamine reductase*

Hydroxylamine reductase was first observed by Taniguchi *et al*. (1956) in bacteria by means of artificial dye carriers, but they did not characterize the natural constituents of the system. Subsequently Zucker and Nason (1955) identified the enzyme in *Neurospora* and in soybean leaves, and showed that it was metal- and flavin-dependent. Recent work in this laboratory has shown the enzyme to be markedly reduced in extracts from felts deficient in Mn. Other trace-metal deficiencies were without effect on the enzyme. This enzymic step does not appear to involve a phosphorylation as it is unaffected by uncoupling reagents (Medina and Nicholas (in press)).

Klausmeyer and Bard (1954) have found ammonia dehydrogenase in

Bacillus subtilis which reversibly catalyses the formation of ammonia and DPN from DPNH and hydroxylamine.

The basic assumption in the aliphatic reduction sequence is that two electron changes are involved in each enzymic step, which implies an intermediate having an oxidation level for N of +1. This is now supported by direct experimental proof of the existence of hyponitrite reductase in plants (Medina and Nicholas, 1957*b*). The aliphatic series may be summarized as follows:

Electron donor	TPNH or DPNH →								
Cofactor	FAD →								
Metal constituent		Mo		Fe Cu		Fe Cu		Mn	
Oxidation or reduction state of N	+5		+3		+1		−1		−3
Sequence	nitrate	→	nitrite	→	hyponitrite	→	hydroxylamine	→	ammonia
Phosphorylation		—		P		P		—	

Aromatic route

An organo-reductive pathway in plants was suggested by Haba (1950) and later by Silver and McElroy (1954), who showed that mutants of *Neurospora* unable to grow on nitrate or nitrite contained enzymes that reduced nitrite and hydroxylamine to ammonia. They also found some mutants that grew normally on ammonia as the sole N source required an exogenous supply of pyridoxine to grow on nitrite or nitrate. They suggested that pyridoxine phosphate combines with hydroxylamine to form pyridoxamine phosphate, which is then transaminated via a keto acid to form amino-acids.

Zucker and Nason (1955) identified a metal- and flavin-dependent *m*-dinitrobenzene reductase in *Neurospora* but this may only be a detoxication mechanism as the incorporation of *m*-nitroaniline into protein was not shown. The addition of *m*-dinitrobenzene to the media is necessary before the enzyme is formed. At the present time the evidence for the aliphatic reduction route is more complete.

NITROGEN FIXATION

During the fixation of atmospheric nitrogen by free-living bacteria, e.g. *Azotobacter* and *Clostridia*, and by root nodule bacteria, e.g. *Rhizobia*, a Mo requirement has been shown which can be partially replaced by V (Bortels, 1930; Horner *et al.*, 1942). In this laboratory Fe and Mo were shown to be essential for N_2 fixation by *Azotobacter* and *Clostridia* and in some strains of the former V and W can partially replace Mo in this process. The precise roles of metals in N_2 fixation are still not clear although recent enzymic studies may provide useful clues.

Hydrogenase

Stephenson and Stickland (1932) first showed that several heterotrophic species of bacteria could reversibly activate H_2; the enzyme responsible was called hydrogenase. Lee, Wilson, and Wilson (1942) showed that hydrogenase activity in *Azotobacter* was linked to the nitrogen-fixing system. Cells grown with combined nitrogen had considerably less enzyme activity than those given N_2 only. Koffler and Wilson (1951) have suggested that N_2, adsorbed by a nitrogenase enzyme (not characterized), is reduced by the hydrogenase system. This hypothesis is not compatible with the following observations: (*a*) the enzyme is present in bacteria which require combined nitrogen for growth, e.g. *Escherischia coli*, (*b*) in experiments made in this laboratory in which the following valency states, Mo^{6+}, Mo^{5+}, Mo^{3+} and V^{5+}, V^{4+}, and V^{3+} were supplied to *Azotobacter*, hydrogenase activity in the V series was comparable with that in the Mo group but N_2 fixation was much reduced when V was substituted for Mo (Table VII).

TABLE VII

The effect of Valence states of Molybdenum and Vanadium on Mo *and* V *uptake*, N_2 *fixation, and hydrogenase activity of Azotobacter vinelandii* (*O strain*)

(5 litre cultures)

Valence state of* Molybdenum or Vanadium	Mo^{6+}	Mo^{5+}	Mo_3^+	V^{5+}	V^{4+}	V^{3+}	omit both Mo and V
Total Mo or V uptake (μg.) .	579	558	300	206	271	966	not detected
Total N_2 fixed in cells (mg.) .	318	265	180	160	135	83	83
Hydrogenase (μ litres H_2 fixed per mg. protein per hr.) .	920	840	1,270	1,130	955	1,010	877

* 1 mg. of each valence state of either Mo or V per 5 litres of culture solution.

Shug *et al.* (1954) have shown that hydrogenase from *Clostridium pasteurianum* is a molybdo-flavoprotein and that the metal is required for the reduction of cytochrome c. In experiments in this laboratory with both *Azotobacter* and *Clostridium*, iron deficiency had a far greater effect in reducing hydrogenase than did a shortage of molybdenum. Mo deficiency, however, depleted N_2 fixation but hydrogenase activity was not markedly reduced; Fe deficiency depleted both systems. Thus hydrogenase action is not necessarily coupled to N_2 fixation. Waring and Werkman (1952) have also shown that Fe is required for the formation of hydrogenase in *Aerobacter aerogenes* and Gest *et al.* (1956) demonstrated that the enzyme was Fe-dependent and they proposed the following scheme for hydrogenase action:

$$\begin{tikzcd} H_2+\text{Enzyme }(\text{Fe}^{++}) \arrow[r, shift left] & \text{Enzyme }(\text{Fe}^{++}) : 2\text{H} \arrow[l, shift left] \arrow[r, shift left] \arrow[d, "(\text{flavin})"] & \text{Enzyme Fe}^{++} - \text{flavin} : 2\text{H} \arrow[l, shift left] \arrow[d, "\text{Mo}"] \\ & \text{Methylene blue} & 2 \text{ methyl viologen electron} + 2\text{H}^{+} \end{tikzcd}$$

According to their scheme molecular hydrogen is activated by a ferrous enzyme to form a reduced enzyme complex which can be oxidized through a flavin component to methylene blue or similar two electron acceptors. Alternatively Mo could combine with the enzyme and the reduced flavin could be reoxidized by one electron acceptor such as the methyl viologen dyes or by cytochrome c. The reduction of the viologens by H_2 and the formation of hydrogen from reduced methyl viologen dyes would require flavin and the two metals. Should Mo be absent then hydrogenase would react with two electron acceptors only, e.g. methylene blue. In support of this scheme it has been shown that in hydrogenase from *Clostridium pasteurianum*, Mo is required for the reduction of cytochrome c but not for methylene blue reduction. The precise role of metals in hydrogenase is still obscure; Fe has been confirmed as a constituent of it but the molybdenum requirement is still in doubt. By analogy with nitrate reductase (Nicholas and Stevens, 1955), it is likely that the metals undergo valency changes during hydrogenase action.

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Fusarium Wilt of Bananas in Jamaica

III. Attempted Control

BY

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ABSTRACT

Experiments were set up in an attempt to control wilt in 'Gros Michel'. Passive measures of eradication and quarantine seldom achieved more than temporary results. Soil disinfection by fumigants was unpromising. Flood-fallowing, important in Central America, was not practicable in the West Indies. Application to the soil of lime, phosphate, and potash, separately or in combination, had little or no effect on wilt incidence; application of nitrogen often resulted in an earlier appearance of wilt. Supplements of various organic materials such as green manures, compost, and bagasse also proved ineffective. Possible reasons for these failures are discussed, and various suggestions are made for the prevention or limitation of wilt in resistant varieties.

INTRODUCTION

WHEN the current investigation began early in 1950 it was still uncertain whether the wilt-resistant 'Lacatan' could satisfactorily replace 'Gros Michel' as the main export variety, and no suitable hybrid banana was forthcoming. Later, even when 'Lacatan' was being shipped in quantity, its disadvantages were such that many growers would have returned to 'Gros Michel' had it been possible to control wilt at all easily. From the outset, therefore, it was desirable to persevere with attempts at direct control although earlier work and current opinion (Baker and Simmonds, 1951) both suggested that success was unlikely. Attention will be confined here to work on 'Gros Michel' over a period of about 4 years, description of the experiments, which from the practical viewpoint were unsuccessful, being chiefly justified by the extreme scarcity of published results. Occasional references to numerous abortive attempts at control are scattered in the literature, and by 1950 only two promising methods had been outlined, at least for 'Gros Michel': one involved treatment of soil with lime and green manures (Scarseth, 1945) and the other soil disinfection by flood-fallowing (Wardlaw, 1947). More encouraging progress towards control of banana wilt is being made in the breeding of resistant varieties. The theory of banana breeding, as described by Dodds (1943), is little changed, but its practice has advanced considerably under the direction of Mr. N. W. Simmonds, Senior Cytogeneticist of the West Indian Banana Research Scheme. The chief advances have arisen

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through collection and synthesis of new clones of *Musa acuminata* Colla which are disease-resistant and have good enough bunch and fruit characters to be worth using as male parents.

PASSIVE CONTROL MEASURES

The history of attempted control of banana wilt in Jamaica by eradication and quarantine is well summarized by Garrett (1944). In the light of earlier events it is instructive to record experience at the Banana Breeding Station, Bodles, where 'Gros Michel' was grown on land not formerly planted with bananas. At first, wilt cases were given a '9-root' treatment in which the affected plant and eight surrounding it were destroyed and an isolation trench dug. In places where wilt was occurring regularly, the first cases to arise immediately outside the trenches occurred 6 to 8 months later, probably as soon as they would have done in the absence of any treatment. Later, when the breeding programme demanded maximum conservation of 'Gros Michel', treatment ('1-root') was confined to affected plants. Once again, wilt often occurred in adjacent plants within a few months, and the situation was not improved when corrugated metal sheeting was placed in the trench to provide a continuous barrier around the site of the diseased plant. Furthermore, it proved difficult effectively to operate such methods owing to the need for frequent irrigation. Although radial spread of wilt is sometimes temporarily checked by drainage ditches, trenches dug after wilt has appeared with the intention of preventing such spread generally prove ineffective, even when made deep enough to sever all lateral roots.

There are several possible reasons for the failure of such measures. Firstly, it is now known that in any outbreak from a third to a half of all wilt cases are 'spontaneous', arising independently of pre-existing ones, and that these may appear at a fairly steady if slow rate for several years after planting. The most to be expected from passive measures, therefore, is a reduction in the incidence of secondary cases near spontaneous ones. Moreover, with a proportion of spontaneous cases, averaging about 20 per cent. in six plantations for which records were kept, two adjacent plants wilt within a few weeks of each other. In such instances measures confined to the plant first affected in no way alter the course of disease in the adjacent one. Even with plants developing wilt 2 months or more after their neighbours, cases which may reasonably be regarded as secondary, such '1-root' treatments usually fail. It seems probable that such plants are often critically infected before their neighbours wilt, which would explain the earlier condemnation of '1-root' methods and the belief that a '9-root' treatment constituted the safe minimum. This rapid spread of disease may be due to the fact that rootlets through which initial infections occur decompose very rapidly and that infected main roots often begin to rot a month or so later: thus the pathogen must often be released into the soil locally before a plant wilts. The pattern of wilt development in plantations strongly suggests a previous gradual build-up and cryptic spread of the pathogen in the soil, and its presence was in fact detected by means of a glasshouse test (Rishbeth, 1955) in a plantation where 'Gros Michel' had grown for 5 years without suffering wilt. By contrast, it was generally believed at the time of the earlier outbreaks that the presence of *Fusarium oxysporum f. cubense* (E. F. Smith) Snyder and Hansen was invariably correlated with that of wilt, and that the main problem was to check radial spread. In retrospect, it seems likely that any failure was due at least as much to the incidence of new spontaneous cases as to incomplete prevention of secondary ones.

These considerations imply that such control measures are in the long run often powerless to check outbreaks of wilt, but not that they are without value. Although on some soils resistance of 'Gros Michel' to wilt is so low that the disease is likely to develop almost as soon as the pathogen arrives, on others there is considerable resistance and wilt may develop only when a certain critical level in soil population of the pathogen is reached. How soon this level is attained depends, amongst other things, upon the rate at which the pathogen is introduced from elsewhere, for instance by flooding, and the rate of build-up from initial cases of wilt. Although adequate controls are seldom available in commercial plantations, it may be noted that in recent years wilt has apparently been prevented from spreading on many soils of medium to high fertility by prompt control measures. Conversely, experiments show that when infected banana tissue is scattered around 'Gros Michel', much as occurs with neglected cases of wilt, onset of the disease is hastened (Rishbeth, 1957). The current investigation amply confirmed earlier beliefs about the importance of infected plants and contaminated tools as potential agents in the local build-up and dispersal of *F. oxysporum f. cubense*.

The heavy gas-oil treatment devised by Smith (1932) for killing affected plants *in situ* represented a big improvement over earlier methods of destruction, not least in avoiding disturbance to the soil. However, this treatment is sometimes followed by a troublesome regrowth of suckers and therefore observations were made on the effect of hormone weed-killers. In one method the main shoot and all suckers were cut and the exposed surfaces treated at once with about 1 pint of gas oil containing 2 per cent. 2,4-D. After 2 months growth of new suckers was very slight. In another method the standing plant was injected with about 30 ml. of 2,4,5-T by means of a hypodermic syringe. Plants died rapidly but a slight growth of suckers followed. Injection of 2,4,5-T into banana *stumps* failed to check sucker growth. Methods for killing banana stools have now been described in New South Wales (Jeater, Cann, and Eastwood, 1951) and in Tanganyika (Wallace, 1952). It is important to realize that death of banana tissues does not ensure destruction of the pathogen: *F. oxysporum f. cubense* was detected in a banana rhizome 3 weeks after successful treatment with gas oil.

ACTIVE CONTROL MEASURES

(i) *Soil disinfection*

Since space was limited at the Banana Breeding Station, Bodles, 'Gros Michel' was replanted on the sites of former wilt cases after the soil had been treated. Soil disinfection was attempted by injecting at points 18 in. apart and at a depth of 6 in., first with carbon disulphide and later with 0·8 per cent. formalin. Thorough mixing of soil with 0·8 per cent. formalin was also tried. In all instances 'Gros Michel' developed wilt, the average interval after treatment with carbon disulphide being 7 months and that after treatment with formalin 5 months.

A few pilot experiments were carried out to determine whether the efficiency of such small-scale methods could be improved. The effect of various treatments on the soil microflora was tested by plating, using the direct method of Warcup (1950): this, though no substitute for field trials with 'Gros Michel', was considered adequate for screening purposes. It was soon apparent that the methods used at Bodles were erratic in effect. Soils, mainly light to medium loams, at various moisture contents were injected at points 12 in. apart and at a depth of 6 in. with various fumigants, including 4 per cent. and full-strength formalin, technical chlorobromopropene, and an organo-mercury compound. The soil microflora was little affected except in the immediate vicinity of the point of injection. Small plots were then watered with 4 per cent. formalin, 1 gallon being applied per square yard, but this also proved ineffective unless evaporation was prevented by immediate flooding. When flooding was repeated several times to wash the formalin down, the soil microflora was markedly affected to a depth of 12 in. Under similar conditions 0·8 per cent. formalin had little effect. Since the cost of this treatment even on a small field scale would have been prohibitive, no further tests were made.

These results amply justify the adverse comments of Smith (1932*b*) on direct control and are possibly to be explained by the high soil temperatures. Some of the effects obtained with formalin paralleled those described in England by Warcup (1951). In untreated soils, *Aspergillus* spp. and especially *A. niger* van Tiegh. were abundant and *Fusarium solani* (Mart.) Appel and Wollenw. emend. Snyder and Hansen was moderately abundant; *Trichoderma viride* Pers. ex Fr. was less common. After the most effective treatments the soil was virtually sterile and remained so for 2 weeks; generally, however, *T. viride* became dominant within a week or so of formalin application. This dominance of *T. viride* sometimes lasted as long as 3 months, but commonly other fungi recolonized the soil within this period.

For nearly 20 years flood-fallowing has been used in parts of Central America to eradicate *Fusarium oxysporum f. cubense* from sites where 'Gros Michel' has succumbed to wilt. It was difficult to find suitable sites for flooding in Jamaica and unfortunately one experiment had to be abandoned on account of hurricane damage. Observations were limited to a small, heavily infested plot at Bodles which was flooded for 9 months, though it was impossible to maintain water at a depth of more than 6 in. At this freely draining site conditions cannot have been sufficiently anaerobic to destroy the pathogen (Stover, 1955) and 'Gros Michel' developed wilt a few months after replanting. Soil fungi had decreased in numbers, the count at 1–6 in. depth being only 25 per cent. of its former value (cf. Stover, 1953), and the composition of the microflora had changed, a species of *Chaetomium* being a prominent survivor. A further point of some interest, in view of the relation between wilt incidence and soil fertility (Rishbeth, 1957), is that available potash in the first 6 in. of the soil profile had declined from 990 to 260 p.p.m. Even if successful on an experimental scale, flood-fallowing could hardly be adopted in the West Indies. Suitably level land is scarce and, where available, is often very freely draining. Other serious drawbacks are the very high initial cost and the necessity for copious supplies of water, the lakes mentioned by Stover (1953) requiring an inflow of 8 cu. ft. of water per second.

(ii) *Addition of inorganic fertilizers to the soil*

In 1948 there were no published records of controlled experiments to test the effect of inorganic fertilizers on wilt incidence. Dr. S. D. Garrett, at that time Plant Pathologist of the West Indian Banana Research Scheme, accordingly set up a comprehensive experiment to determine the effect of adding nitrogen, phosphate, potash, and lime, and all combinations of these fertilizers, to 'Gros Michel'. This was carried out on various soil types at five sites where bananas had earlier been severely affected by wilt. Application of lime differed with the site, the amount added to the appropriate plots being calculated to bring the pH to 7·8: at one site no application was necessary. Nitrogen was applied as sulphate of ammonia, the monthly dose of 4 oz. per plant being equivalent to an application of 9·6 cwt. per acre per year. At 3-monthly intervals the appropriate plants received 7·5 oz. superphosphate (≡ 6 cwt. per acre per year) and 6·5 oz. muriate of potash (≡ 5 cwt. per acre per year). With the untreated control there were in all 16 treatments, each of which was repeated twice: the plots each contained 20 plants, surrounded by a single guard row. Wilt incidence was recorded at monthly intervals and the height of all healthy plants was measured every 2 months. For the five sites Table I records soil texture, reaction, available phosphate and potash, and the percentage incidence of wilt in all plots 16 months after planting.

TABLE I

Soil Conditions Existing at Sites Selected for a Fertilizer Experiment, and Wilt Incidence in All Plots after Sixteen Months

	Soil conditions				
Site	Texture	pH	p.p.m.P_2O_5	p.p.m.K_2O	Percentage of wilted plants
1	Stiff clay	5·7	61	257	56
2	Sandy loam	8·1	132	100	27
3	Silty loam	8·6	478	290	56
4	Light loam	7·8	376	51	32
5	Stiff clay	5·4	10	151	26

Locality of sites:
1. Orange River (A), St. Mary. 2. Caenwood (A), Portland. 3. Twickenham Park, St. Catherine. 4. Holland, St. Thomas. 5. Amity Hall, St. James.

The rate at which wilt developed differed with the locality, and at sites 4 and

and 5 it varied considerably from one set of replicate plots to the other. At no site did any treatment result in a marked increase of resistance. In the more heavily affected section at site 4 one plot which received potash only was attacked comparatively slowly, nine plants having wilted after 18 months, whereas in other plots nearly all had wilted by this time. The effect of potash was not entirely consistent at this site, however. The main feature was the tendency for wilt to appear earlier in plants fertilized with nitrogen. Table II records for four sites the number of wilted plants in plots receiving and not receiving nitrogen and the average height of plants in these two sets of plots. Observations were made 8 to 10 months after planting. In this experiment the earlier onset of wilt in plants receiving nitrogen was correlated with their faster growth-rate, the effect being particularly marked at sites 1 and 4. However, a similar effect may occur in the absence of any marked difference in growth-rate, and other experiments (Rishbeth, 1957) suggest that nitrogen fertilizing leads to more rapid growth of the pathogen in the plant.

TABLE II

Wilt Incidence and Height of 'Gros Michel' Receiving and not Receiving Nitrogen

Site	No. of wilt cases (out of 320)		Mean height of plants (ft.)	
(as in Table I)	−N	+N	−N	+N
1	57	194	2·9	5·4
2	22	31	9·4	10·3
3	69	98	5·5	6·2
4	7	42	3·4	5·6

In the light of observations in other 'Gros Michel' plantations, the disappointing result of this experiment is at least partially understandable. Two of the sites were on eroded, acid clays characteristic of much land formerly under bananas, where for a susceptible variety no amount of fertilizing could be expected adequately to offset poor rooting conditions. Another difficulty with such sites is to achieve effective liming: in this instance fairly heavy applications of marl had failed to affect soil pH appreciably after 2 years. The remaining three sites were on alkaline loams, alluvial in origin, of very light to medium texture and fairly rich in available phosphate but medium to poor in available potash: as compared with alluvial soils bearing healthy plantations, the texture tended to be lighter and available potash much lower. Analysis of samples taken 1 year after the final application of fertilizer showed that the soil of plots receiving potash was little or no richer in available potash than at the beginning of the experiment.

(iii) *Addition of organic materials to the soil*

In view of the poor promise shown by inorganic fertilizing, it was decided to test the effect on wilt incidence of adding various types of organic matter to the soil. Scarseth (1945) had reported the beneficial effect of turning in green manures after adequate liming of acid soils. It also seemed possible that addition of organic matter might improve the moisture-holding capacity of light-textured soils sufficiently to induce increased resistance, a hope encouraged by glasshouse experiments with bagasse. Trials were initiated at six sites with a variety of treatments. The layout resembled that in the previous experiment but was on a smaller scale, the treatments selected for a particular site being replicated but not combined factorially. The following materials were forked into the first 12 in. of soil before planting, the amounts being expressed as tons per acre: compost (25–65), coir (25), and bagasse (15–25). The compost was of various origins, the most satisfactory probably being that derived from coir. Symptoms of nitrogen shortage which sometimes appeared for the first month or two, especially with bagasse, were ignored for the purposes of this experiment. For green manures various leguminous crops were grown and turned in just before flowering, two successive crops of *Canavalia ensiformis* DC. representing the minimum treatment before planting. With half the plots further crops were grown, either more *Canavalia* or a species of *Crotalaria* (*C. juncea* L. or *C. usaramoensis* E. G. Baker): this preliminary treatment lasted about one year. A proportion of the plots were left with a cover-crop, either *Indigofera endecaphylla* Jacq. or *Pueraria phaseoloides* Benth. Some characteristics of the sites and the percentage incidence of wilt following the treatments are given in Table III. Once again the rate at which wilt developed differed considerably with the site and the records of wilt incidence quoted were obtained at a stage providing the best comparison between plots: this varied but was always within 1 to 2 years of planting. Sites 7 and 11 were low in available phosphate and all plots received an initial dressing of superphosphate equivalent to 4 cwt. per acre.

TABLE III

Soil Conditions Existing at Sites selected for an Experiment with Organic Supplements, and Wilt Incidence after Various Treatments

Soil conditions					Percentage of wilted plants following various treatments
Site	Texture	pH	p.p.m. P_2O_5	p.p.m. K_2O	
6	Clay loam	5·4	183	395	Control (75), compost (73), green manures (85)
7	Clay loam	8·2	26	170	,, (80), ,, (60), ,, (63)
8	Medium loam	8·3	68	264	,, (14), ,, (30), coir (23)
9	Silty loam	7·4	85	474	,, (44), bagasse (33)
10	Sandy loam	8·3	174	142	,, (35), ,, (18)
11	Clay loam	6·8	16	179	,, (38), ,, (8)

Locality of sites:

6. Orange River (B), St. Mary.
7. Williamsfield, Portland.
8. Caenwood (B), Portland.
9. Bodles, St. Catherine.
10. Caymanas, St. Catherine.
11. Pleasant Hill, St. Thomas.

Bananas receiving bagasse tended to grow rather slowly at first and so it is hardly surprising that at any given time wilt incidence in bagasse plots was always less than in the controls. This situation, the converse of that occurring with nitrogen fertilizing, was of course no gain in that flowering was correspondingly delayed. Allowing for such differences in growth-rate, which also occurred between some duplicate plots, it cannot be claimed that any treatment had a marked effect on wilt incidence.

At each of these sites the effect of potash applications was further tested. Although in the earlier experiment applications had been fairly heavy by normal standards it was conceivable that their effect had been too transient to increase wilt resistance. Since preliminary trials showed that, weight for weight, sulphate gave higher values of available potash than muriate, both fertilizers were tested, separately and in conjunction with organic supplements. Applications of potash, given just after planting and thereafter at 2-monthly intervals, were equivalent to 10 cwt. per acre per year for muriate and from 10 to 12 cwt. per acre per year for sulphate. These applications were undoubtedly effective in raising the level of available potash, values of 700–1,500 p.p.m. being maintained throughout the experiment. As before, however, there was no effect on wilt incidence.

At site 6, which had a heavy, acid soil, an attempt was made to increase the effectiveness of liming. Slaked lime was substituted for marl and was incorporated in all plots to a depth of 18 in., the application being equivalent to 12 tons per acre. To conform with Scarseth's type of treatment, all plots were limed, one set 6 months and another set 12 months before planting. About 9 months after the application the effect on soil reaction was somewhat erratic, but the pH at 6 in. and 12 in. depth was usually above 7. Wilt incidence in these plots (Table III), recorded after only 12 months, was generally high: by contrast, growth was so retarded in two adjacent unlimed areas that wilt had scarcely appeared by this time.

At site 6 one plot was exceptional in that after 2 years a group of six plants was still healthy and bearing good fruit. In this small area the soil had a pH of 8·0 and available potash was unusually high, through dumping of fertilizers and other materials for distribution to other parts of the site. Whether these changes had arisen only since the initiation of the current experiment is unknown, but the situation was of interest in providing the nearest parallel in these experiments to the high wilt-resistance of 'Gros Michel' on the very fertile sites of former sugar factories (Rishbeth, 1957). It suggests that if ameliorative treatment is given long enough, perhaps over a period of several years, a certain degree of wilt-resistance might be induced in 'Gros Michel'. In the current investigation time unfortunately did not permit such extended trials, and in any case prolonged treatment would be unacceptable to planters.

DISCUSSION

In the foregoing experiments there was a slight risk of reintroducing *F. oxysporum f. cubense* with planting material, despite thorough inspection. However, conditions were representative in the sense that similar material would have had to be used in any rehabilitation of 'Gros Michel'. In the trials with organic supplements site 6 was supplied with planting material from the same source as site 8, and thus the effect of such reintroduction on wilt incidence is likely to have been small.

Failure of organic supplements to decrease wilt incidence corresponds with the experience of planters who returned to cultivation of 'Gros Michel' without success on soils which had borne sugar-cane for many years and therefore had a high initial content of organic matter. On the other hand, regular application of organic matter as a mulch has probably been instrumental in keeping many plantations free from serious wilt, particularly in the hill country of central Jamaica. Green manuring often causes marked temporary changes in the soil microflora, but there is no evidence that it reduces the population of *F. oxysporum f. cubense* through antagonistic effects, as suggested by Thorold (1945). Further, the type of soil microflora appears to have little influence on root infection by the pathogen. In a glasshouse experiment with 'Gros Michel' of the type described earlier (Rishbeth, 1957), addition to the soil of 5 per cent. by weight of a green manure led to no reduction in the number of rhizome infections.

Perhaps the greatest cause for disappointment lies in the failure of potash fertilizing to decrease wilt incidence (as it often does with *Fusarium* wilt of cotton), even where soil conditions for growth of 'Gros Michel' appear to be optimal. It is likely that this variety, to achieve significant wilt-resistance, must take up more potash than is necessary for normal growth and fruit production. The critical difference between the behaviour of 'Gros Michel' on soils naturally rich in potash and on poorer soils replanted after wilt and heavily fertilized may possibly arise from a lower uptake of potash in the latter, from a higher soil population of the pathogen, or from a combination of these factors. With some plants uptake of potash is impeded by poor aeration (Hammond, Allaway, and Loomis, 1955), and this factor may well be important, for instance, in some heavy clays and compact, silty loams. In this connexion it is probably significant that 'Gros Michel' resists wilt most successfully on soils that are not only rich in potash but also well aerated. Although banana wilt is a conditional disease (Wardlaw, 1930) in the sense that root infections with *F. oxysporum f. cubense* do not invariably lead to collapse of the plant, it is now clear that short-term measures of soil improvement cannot be expected to affect its incidence in replanted 'Gros Michel'. Further, even if some generally applicable method were found of controlling the disease in this variety, it would probably not compete economically with the growing of a resistant one.

Since 'Lacatan' can be grown successfully and free from wilt on a wide range of soils, except very occasionally at sites similar to Orange River (A), there seems little point in pursuing further the type of investigation described above. In areas where the situation allows, a more promising field for any future work might concern the conditions necessary to keep existing healthy 'Gros Michel' plantations free from wilt. It may well be easier to prevent wilt from developing at sites where resistance to it is initially high than to control the disease once the soil population of *F. oxysporum f. cubense* has reached a dangerous level. Otherwise, important advances in knowledge of wilt-resistance seem most likely to arise from work on banana physiology, especially, perhaps, on root growth and potash uptake.

Although with respect to wilt incidence resistant varieties can tolerate a far wider range of soil conditions, indefinite freedom from wilt can by no means be taken for granted. In the light of knowledge derived from this and earlier investigations, the following points may be made about prevention or limitation of the disease in such varieties. Firstly, selection of site is important. In the Caribbean area generally, sites are planted at which soil conditions are entirely unsuitable for bananas, and in Jamaica itself many sites where severe erosion has followed earlier banana cultivation have been replanted with 'Lacatan'. Such conditions encourage an early breakdown of resistance. Attention should be paid to maintenance of soil structure and fertility, especially where continuous banana cultivation is the rule. Poor drainage, leading to temporary water-logging, is the most frequent cause of breakdown in wilt-resistant varieties. Balanced fertilizing is also important, unduly heavy applications of nitrogen being likely to promote wilt. Finally, for testing wilt-resistance in new varieties, too much reliance should not be placed on heavy inoculum alone: after preliminary screening, promising varieties should undergo trials on a range of soil types, including some known to favour wilt.

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The Widespread Occurrence of Polyphosphate in Lower Plants

BY

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With Plates XXVI and XXVII

ABSTRACT

The widespread occurrence of polyphosphate (PoP) in lower plants is described. PoP granules have been cytochemically detected in algae, slime moulds, and mosses. A systematic survey is given of lower plants in which the occurrence of PoP has been demonstrated with both the present studies and previous investigations.

The localization of PoP in cells has been studied. PoP granules have been observed to be present in the cytoplasm, the cytoplasmic vacuole, and in chloroplasts of different algae. In Zygnemataceae and in mosses PoP granules are localized in the chloroplasts which also contain pentose nucleic acid.

A relationship between PoP, pentose nucleic acid (PNA), and growth has been found and is discussed. A possible *in situ* synthesis of PoP in chloroplasts from phosphorylated intermediates of the photosynthetic process is suggested.

I. INTRODUCTION

THE presence of polyphosphate (PoP) in plants was first demonstrated by Macfarlane (1936) when she identified PoP as a constituent of the nucleic acid fraction in yeast. Later Jeener and Brachet (1944) observed increased basophilia in yeast grown in a phosphate-containing medium. This basophilia was found independently by Wiame (1946) and Schmidt, Hecht, and Thannhauser (1946) to be due to PoP. Houlahan and Mitchell (1948) demonstrated high amounts of an acid soluble and acid labile inorganic phosphate compound in *Neurospora* mutants. The authors assumed that this compound is identical with the polyphosphates isolated from yeast by Wiame (1946). Mann (1944) has also reported the occurrence of PoP in *Aspergillus niger*. We know of the presence of PoP in bacterial cells by the works of Wiame (1947*a*), Winkler (1953), Macary (1951), Ebel (1952*a*, 1952*b*), and Davis and Mudd (1954). PoP in the blue-green alga *Phormidium* had been demonstrated as granules by Ebel (1952*a*) using a newly developed cytochemical test, Albaum, Schatz, Hutner, and Hirschfeld (1950) isolated PoP from *Euglena*, and Stich (1953) found high amounts of PoP in *Acetabularia*.

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Sommer and Booth (1938), using an unusual physical method, reported the finding of PoP in *Chlorella*.

In reviewing the above-listed plants one finds that they represent a variety of taxonomical groups. This fact would strongly suggest that PoP might occur generally in lower plants. Further, volutin, a substance of hitherto uncertain chemical composition, has been reported to occur in many algae. Since volutin is characterized by cytochemical reactions also given by PoP, it has been regarded as equivalent to PoP although often no experimental proof of this has been given.

In the present studies many algae have been examined for the occurrence of PoP as well as mosses, ferns, and gymnosperms.

II. METHODS

Polyphosphate (PoP) gives a metachromatic reaction on staining with toluidine blue (Wiame, 1947*b*) while nucleic acids, DNA and PNA, stain orthochromatically.[1] Staining at very low pH (Lindegren, 1951) or staining under near neutral conditions followed by treatment with 0·1-N . HCl (Wiame, 1947*a*) removes the colour from nucleic acids and only the metachromatic material retains a characteristic red or purple colour. The metachromatic reaction at low pH is also given by certain sulphuric esters of polysaccharides (Lison, 1935*a*, 1935*b*). These compounds do not interfere with the cytochemical demonstration, however, since the PoP responsible for the metachromatic reaction is completely extracted from fixed material with cold 10 per cent. trichloracetic acid (TCA) while acid polysaccharides remain unaffected by this treatment. PoP can also be extracted from unfixed cells by hot water (Grimme, 1902; Lindegren, McClary, and Williams, 1955). It has also been shown (Stich and Keck, in the press) that incubation with lead or barium salts at low pH prior to staining decreases the dye-binding capacity of PoP, whereas nucleic acids and acid polysaccharides stain with toluidine blue as before.

For chemical characterization PoP can be precipitated from TCA extracts as barium salt (Schmidt, Hecht, and Thannhauser, 1946; Wiame, 1947*c*). A solution of isolated PoP gives a metachromatic reaction which can be used for a spectrophotometric estimation of PoP (Albaum *et al.*, 1950; Damle and Krishnan, 1954). Purified PoP is completely hydrolysed with 1-N . HCl after 7 minutes at 100° C.

For the present investigations the following methods were used:

Material was fixed in ethanol-formalin (96 per cent. ethanol–30 per cent. formalin, 5 : 1) and Carnoy, stained with a saturated aqueous solution of toluidine blue (National Aniline Division, New York) for 10 minutes, rinsed briefly in water and then differentiated in 0·1-N . HCl for 2 minutes. After dehydration in alcohol the material was mounted in canada balsam. The same staining procedure was applied to material embedded in paraffin and sectioned 6 μ thick. In each case control slides were treated with 10 per cent. TCA at 4° C. for 6 hours to extract the PoP before staining.

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[1] PNA sometimes gives a weak metachromatic staining which, however, disappears on differentiation with 0·1-N . HCl.

Vital staining with a dilute solution of toluidine blue (10^{-4} molar) was used for detailed study of the localization of the metachromatic granules in the living cell. The material was first examined in a drop of water and a drop of the dilute dye solution was added to the edge of the cover-slip. If the metachromatic staining was masked by an overall blue staining of the cell, a trace of an 0·01-N . HCl solution was added.

Pentose nucleic acid (PNA) was demonstrated cytochemically by staining with gallocyanine according to Einarsen (1952) and with toluidine blue at pH 4·2. Controls were treated with PNA-ase as described by Kaufmann, Gay, and McDonald (1951).

When larger amounts of sufficiently pure material were available PoP was isolated from TCA extracts according to Wiame (1949) by precipitation with barium nitrate. The orthophosphate liberated after 7 minutes' hydrolysis with 1-N . HCl at 100° C. was measured, using the ultramicro method of Krugelis (1951).

III. RESULTS

General observations

The present study deals mainly with algae, although slime moulds, mosses, and ferns were also examined. In the case of the algae the attempt was made to include at least one genus from each order of algae. Unfortunately Rhodophyta and Phaeophyta were not available, but will be examined in the future. It was found that PoP could be demonstrated in all the algae studied.

PoP is by no means a constant constituent of the algal cell. The amount of PoP cytochemically found in cells of different samples of the same material shows great variation, and is probably an expression of the physiological state of the plants. Freshly collected diatoms, for instance, sometimes showed almost no metachromatic material, while in other samples the same forms contained numerous granules per cell. In *Mougeotia*, even cells in the same thread showed noticeable differences in this respect. Such differences in PoP content are more noticeable in the number of granules than in the size. There seems to be an upper limit to the size of the granules, which is commonly characteristic for a given plant. No direct correlation between the content of PoP and the products of photosynthesis (starch, glycogen, oil) could be found. It seemed, however, that more PoP was present in actively growing cells. In *Chara* most PoP is present in young, uncorticated branches. Similarly, in protonema of *Leptobryum* only the young tip cells contain PoP. *Spirogyra*, when kept in tap-water in the laboratory for some time, ceases to grow and accumulates starch in the chloroplasts. At this time the PoP granules became fewer and finally disappeared. More evidence is needed, however, before a general conclusion can be drawn.

The cell-walls of some algae stained so heavily with toluidine blue that a direct observation of the interior of the cells was not possible. In such instances the membrane was cut open and the contents were squeezed out on a slide for fixation and staining, or gallocyanine was used as a substitute for toluidine blue. This dye did not stain cell-walls and gave a weak metachromatic reaction with PoP.

Systematic survey

In Table I are tabulated the results of a systematic survey of the occurrence of PoP in lower plants. It would appear from Table I that PoP is of widespread occurrence in lower plants. In all of the lower plants examined in the present investigations it was possible to show that PoP granules were present at least during some stage or in some part of the plant.

TABLE I

Schizomycophyta (Bacteria)	Mycobacterium	Winder and Denneny (1954)
Myxomycophyta (Slime moulds)	Physarum	Keck and Stich
Eumycophyta (Fungi)	Saccharomyces	Macfarlane (1936), Wiame (1946) Schmidt, Hecht, and Thannhauser (1946)
	Neurospora	Houlahan and Mitchell (1948)
	Aspergillus	Mann (1944)
Cyanophyta	Phormidium	Ebel (1952)
	Oscillatoria	Keck and Stich
Chrysophyta		
Bacillariophyceae	Fragilaria	,, ,, ,,
	Navicula	,, ,, ,,
Heterokontae	Vaucheria	,, ,, ,,
Euglenophyta	Euglena	Albaum *et al.* (1950), Keck and Stich
Chlorophyta	Chlorella	Sommer and Booth (1938)
	Ulothrix	Keck and Stich
	Oedogonium	,, ,, ,,
	Zygnema	,, ,, ,,
	Mougeotia	,, ,, ,,
	Spirogyra	,, ,, ,,
	Cosmarium	,, ,, ,,
	Chara	,, ,, ,,
	Acetabularia	Stich (1953)
Bryophyta	Polytrichum	Keck and Stich
	Leptobryum	,, ,, ,,

In contrast preliminary studies of some higher plants did not detect any PoP in *Polypodium*, *Osmunda* (prothallium, leaf, sporangium), *Psilotum* (stem, sporangium), or *Pinus* (foliage bud, young female cone).

These few negative results do not prove the absence of PoP in all higher plants of course, but exclude a general occurrence, such as appears to be the case for lower plants.

Localization of PoP in the cell

In fixed and stained preparations differentiated with HCl, the PoP granules could easily be detected microscopically. They had an intensive purple colour, whereas all the other cell components were practically unstained. In some instances irregular-shaped granules or even clusters of metachromatic material could be observed which did not correspond to any analogous structures in living cells and therefore must be interpreted as fixation artefacts. Metachromatic particles could also be recognized after vital staining with a very dilute solution of toluidine blue. On longer incubation with this dye structural changes occurred and enlargement of granules or precipitation of PoP in the form of irregular-shaped bodies was observed. These changes proceeded rather slowly so that artefacts could be estimated.

When various materials were studied by the use of both vital and fixed staining procedures, PoP was localized in the cytoplasmic vacuole, the cytoplasm, and in the chloroplasts of different algae. In *Oedogonium*, for instance, PoP seemed to be dissolved in the cytoplasmic vacuole. In living cells cytoplasmic vacuoles appear as optically empty spaces and only seldom were a few very small granules visible. On addition of a dilute toluidine blue solution the granules increased in number and size, and finally the vacuoles were filled with small metachromatic granules, showing vigorous Brownian movement. The ability of toluidine blue to precipitate PoP from solutions has been described by Wiame (1947*b*).

Small PoP granules are present in the cytoplasm of different forms of diatoms (Pl. XXVII, Fig. 11), while in other cases precipitation seemed to have occurred (Pl. XXVI, Figs. 1, 2) in the cytoplasmic vacuole. Some forms of diatoms are attached to a substrate by means of a stalk. Such stalks also give a strong metachromatic reaction. The substance responsible for the metachromasia, however, is not extracted by cold TCA and is therefore probably an acid polysaccharide.

PoP was observed in the cytoplasm of *Chara* in young uncorticated branches. The coenocytic alga *Vaucheria* accumulates PoP in the form of small bodies in the cytoplasm of unfertilized oogonia, whereas the filaments of this plant contain very little PoP. In *Euglena*, where the presence of PoP had been detected by means of chemical analysis (Albaum *et al.*, 1950) we observed numerous metachromatic particles scattered throughout the entire cytoplasm (Pl. XXVI, Fig. 6).

Members of the family *Zygnemataceae* differ in a characteristic way from the other algae in that metachromatic bodies were observed to be situated in the chloroplasts. These chloroplasts contain besides PoP considerable amounts of PNA as indicated by basophilia. In *Spirogyra* we observed small granules (averaging 0·4 to 0·8 μ in diameter) that tend to be arranged in lines which follow the spiral bands of the chloroplasts. The pyrenoids do not contain any PoP but are surrounded by a ring of PoP granules (Pl. XXVI, Fig. 4). After staining for nucleic acids chloroplasts showed a differentiation into a strongly stained narrow central strip which runs lengthwise between adjacent pyrenoids (which are also deeply stained) and a faintly stained background. When only little PoP is present in the chloroplasts it is localized within the PNA-rich central strip (Pl. XXVI, Fig. 5). In cells with more PoP the granules were also found scattered through the rest of the chloroplast. Similar conditions were encountered in *Zygnema*. The PoP granules were found to be somewhat larger in size and fewer in number per cell as compared with *Spirogyra*. They are localized in the outer portion of the star-shaped chloroplasts (Pl. XXVI, Fig. 3). Again a correlation between the site of the PoP granules and PNA could be noticed. In this case the outer layer of the chloroplasts proved to be more strongly basophilic than the central portion which contains the pyrenoid. No PoP granules could be observed near the pyrenoid. *Mougeotia*, the third genus of the *Zygnemataceae*, deviates slightly from the above-outlined scheme. The basophilia of the plate-shaped chloroplasts was less striking. PoP was mainly localized in the chloroplasts but, in rare instances, could also be observed in the cytoplasm (Pls. XXVI and XXVII, Figs. 7, 9).

A localization of PoP granules in chloroplasts was also found in mosses. Investigated were protonema of *Leptobryum* and leaf of *Polytrichum*. A few PoP granules were detected in each of the small chloroplasts (Pl. XXVII, Figs. 8, 13). In the protonema PoP was restricted mainly to cells in the tip of the filaments. The presence of pentose nucleic acid (PNA) in chloroplasts of such cells could be demonstrated by cytochemical reactions.

The presence of nucleoproteins in PoP(-volutin) granules, as often described in the literature, could not be confirmed by our investigations since no basophilia was observed after extraction of the PoP with cold 10 per cent. TCA. In addition, treatment with PNA-ase (dissolved in distilled water, pH adjusted to 6·0) did not alter the metachromatic reaction of these particles. Previous reports to the contrary might be due to buffered enzyme solutions. We found that buffers, even at low pH (citric acid buffer pH 2), gradually extract PoP from fixed cells.

Discussion

Polyphosphate is of widespread occurrence in various groups of lower plants which otherwise differ in many respects, such as nutrition (autotrophic and heterotrophic), photosynthetic pigments, and end-products of photosynthesis (starch, glycogen, oil). In consideration of the recent theories on the physiological role of PoP this finding strongly suggests a common system of energy storage in these plants.

In chloroplasts of certain algae or mosses PoP granules are localized in or near cell structures which contain pentose nucleic acid (PNA). There is no doubt a relationship between PoP, PNA, and growth. In consideration of the importance of PNA and an energy source for protein synthesis it is not impossible that a large part of the protein synthesis in cells of these plants occurs in PNA-rich chloroplasts. The *in situ* presence of PoP could, via the ADP-ATP-system, provide the necessary energy requirements.

In plants where PoP occurs in the cytoplasm (*Acetabularia, Euglena*) PNA also seems to be present mainly in the cytoplasm. However, whether PoP is localized in the cytoplasm or in the chloroplasts, it is mostly concentrated in young actively growing parts of the plants (moss protonema, *Chara*). These facts strengthen the possible relationship between PoP, PNA, and growth.

The formation of PoP in chloroplasts could be explained by the assumption that they are synthesized *in situ* by transphosphorylation reactions directly from phosphorylated intermediates resulting from the photosynthetic process. Several experimental results support this idea. For example, *Chlorella* responds to illumination with a rapid increase in the ATP content (reviewed by Strehler, 1952). The energy gained by these processes could be stored nearby as PoP. In *Acetabularia* no noticeable incorporation of P^{32} into PoP takes place in the dark, whereas on illumination there is a considerable uptake of labelled phosphorus into PoP as shown by autoradiographic methods (Stich, 1955). The PoP formation in relation to photosynthesis in *Chlorella* has been studied by Wintermans (1955). In this excellent paper the possible role of PoP in the photosynthetic process is discussed in detail.

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EXPLANATION OF PLATES

Key to labelling: n = nucleus
p = pyrenoid
v = vacuole
c = chloroplast

PLATE XXVI

Fig. 1. Toluidine blue—HCl. × 1,480. *Navicula*. Dense bodies of PoP in the cytoplasmic vacuoles, probably precipitated during fixation.

Fig. 2. Toluidine blue—HCl. × 990. *Fragilaria*. PoP granules in the cytoplasmic vacuoles.

Fig. 3. Toluidine blue pH 4·2. × 1,320. *Zygnema*. Star-shaped chloroplasts showing peripheric basophilia (PNA) and PoP granules.

Fig. 4. Toluidine blue—HCl. × 1,200. *Spirogyra*. Chloroplasts with PoP granules and pyrenoids. The nucleus is unstained and translucent.

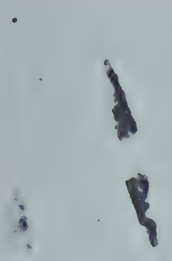
Fig. 5. Toluidine blue pH 4·2. × 1,500. *Spirogyra*. Demonstration of PNA in the chloroplast and pyrenoids. Small PoP granules are here mainly arranged within the PNA-rich central strip between pyrenoids. The nucleus is deeply stained.

Fig. 6. Toluidine blue—HCl. × 1,300. *Euglena*. Numerous small granules are scattered in the cytoplasm. The vacuole is devoid of PoP, the nucleus is unstained.

Fig. 7. Toluidine blue pH 4·2. × 1,320. *Mougeotia*. Band-shaped chloroplast with pyrenoids showing the presence of PoP granules and only little PNA.

PLATE XXVII

Fig. 8. Toluidine blue pH 4·2. × 1,220. *Polytrichum*. Cells of a leaf with basophilic chloroplasts containing small PoP granules.



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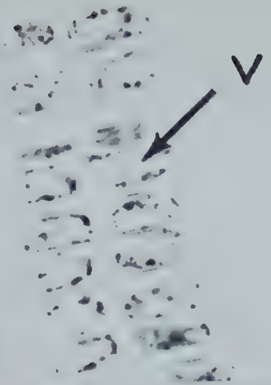

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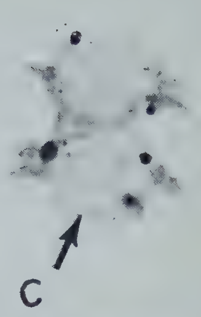

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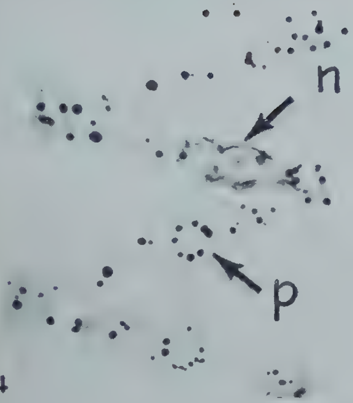

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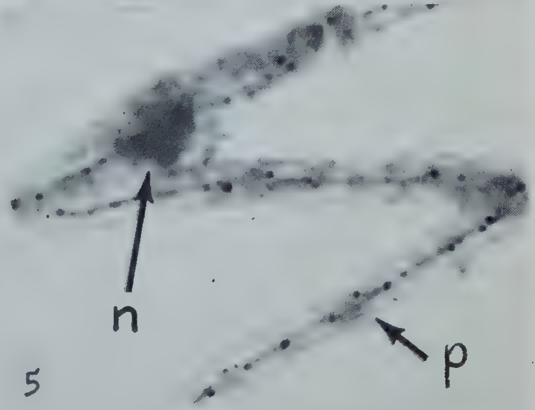

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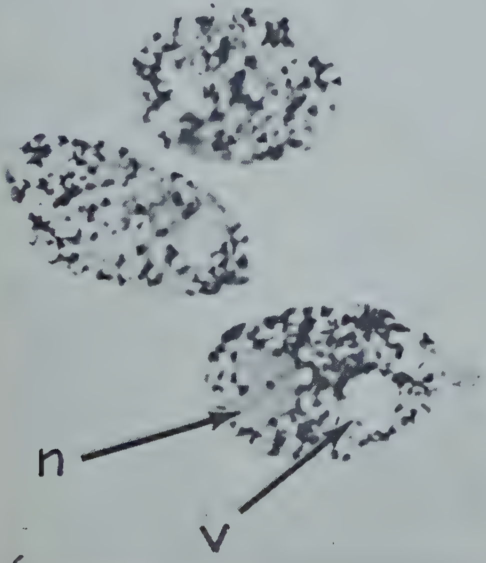

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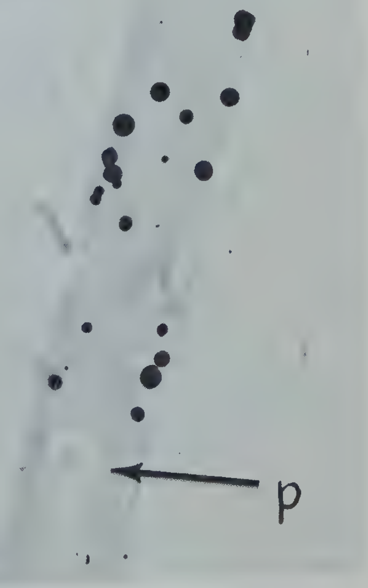

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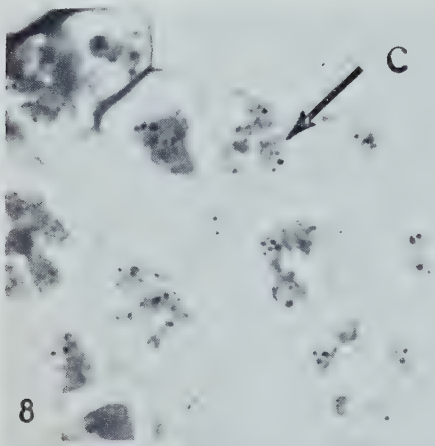
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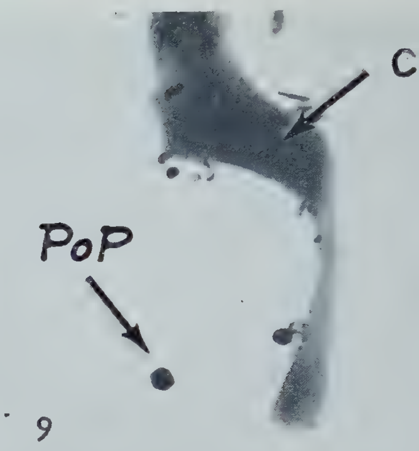
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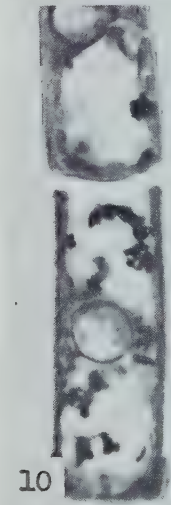
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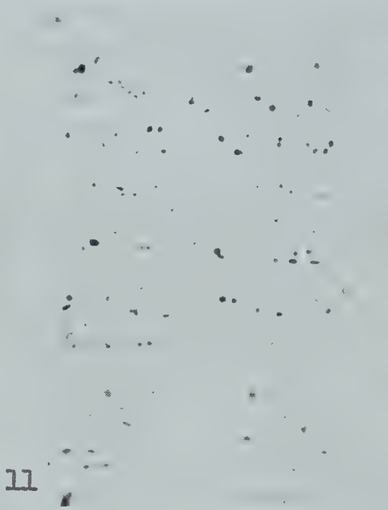
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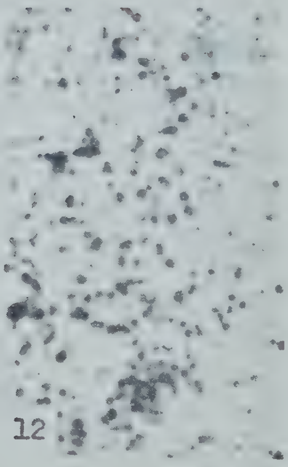
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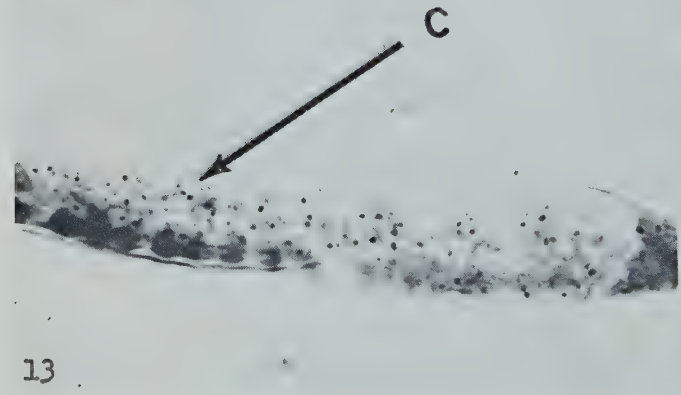
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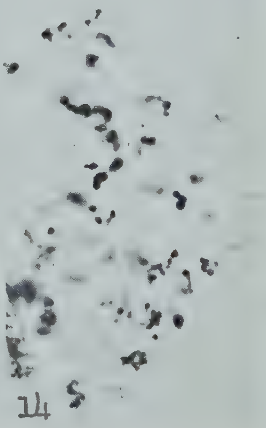
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Fig. 9. Vital staining. × 1,220. *Mougeotia.* PoP granules outside the chloroplast.

Fig. 10. Vital staining. × 1,320. *Oedogonium.* Metachromatic particles in the cytoplasmic vacuoles, due to precipitation with toluidine blue.

Fig. 11. Toluidine blue—HCl. × 1,155. *Fragilaria.* Cytoplasmic PoP particles.

Fig. 12. Toluidine blue—HCl. × 1,155. *Acetabularia.* Cytoplasmic PoP.

Fig. 13. Toluidine blue pH 4·2. × 1,320. *Leptobryum.* Cells of the protonema with many chloroplasts, each containing a few PoP granules.

Fig. 14. Toluidine blue—HCl. × 1,320. *Stentor.* PoP in the symbiontic algae.

Observations on the Aphlebiae and Megasporangia of *Stauropteris burntislandica* P. Bertrand

BY

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With Plate XXVIII and one Figure in the Text

ABSTRACT

The aphlebiae of *Stauropteris burntislandica* are shown to be more complex than the simple forked processes indicated by Surange. They are variable structures and consist of a protuberance or ridge bearing 5–7 appendages probably arising by dichotomous branching; the vascular system is also branched and supplies each large lobe of the aphlebiae with a small strand of tissue of tracheidal character.

A megasporangium is described containing more than four megaspores, possibly as many as seven or eight, indicating that two tetrads may sometimes be involved in megaspore formation.

INTRODUCTION

THE Carboniferous fossil plants *Stauropteris oldhamia* and *S. burntislandica* each consist of a repeatedly branched 'frond', the daughter branches of which arise successively in pairs on either side of the rachis. Situated laterally to the daughter branches near their junction with their parent branch are out-growths, the aphlebiae. It has been often accepted that the aphlebiae are fairly simple structures and they have been indicated as such in reconstructions (Hirmer, 1927; Walton, 1954; Surange, 1952). Some of the published figures and descriptions suggest that at the points of branching there is a pair of aphlebiae, each being an entire, single recurved process (cf. Hirmer, 1927, p. 491, Fig. 580, for *Stauropteris oldhamia*), or a simply forked appendage (cf. Surange, 1952, p. 82, Fig. 5, for *S. burntislandica*). The mode of branching of the 'frond' of *S. burntislandica* has been elucidated in detail by Surange (1952), but relatively little attention was paid to the aphlebiae. The present investigations have shown that at least in *S. burntislandica* the aphlebiae are more complex and variable in structure than some previous descriptions suggest, and that they are subdivided into several elongate lobes, each with its own vascular supply.

MATERIAL AND METHODS

The single block of material used was of calciferous sandstone (Pettycur limestone) from Pettycur, Fife, Scotland. It contained abundant petrified specimens of *Stauropteris burntislandica*. More than 200 serial peel sections

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were taken from several faces of the cut block by the technique of Joy, Willis, and Lacey (1956) as follows. The faces were polished with emery powder, etched in 2 per cent. HCl for 30 seconds, washed and dried. The etched surface was moistened with acetone and a cellulose acetate film 0·05 mm. thick lowered gently on to it; the film was peeled from the matrix after an hour at room temperature or 30 minutes at 34° C. The peels were mounted either permanently in Canada balsam or temporarily in xylol for examination.

For reconstructions of the branching regions of *Stauropteris*, camera lucida drawings showing the outlines of the branches and aphlebiae and of their vascular tissues were prepared from serial sections. These outlines were traced on to sheets of translucent dental wax (during tracing the drawings were covered with a thin sheet of cellulose acetate to protect them from the wax). Tracings were made with a needle which could, by pressure, be used to cut through the wax. The portions so cut out were numbered, stacked in order, and sealed together by means of a very fine gas flame from a glass capillary. The vertical scale of these wax models is the same as the horizontal scale so that the models are in proportion in all dimensions; this was achieved by adjusting the distance between sections (by altering grinding and polishing times) to correspond in scale with the thickness of the wax available (*c.* 1 mm.).

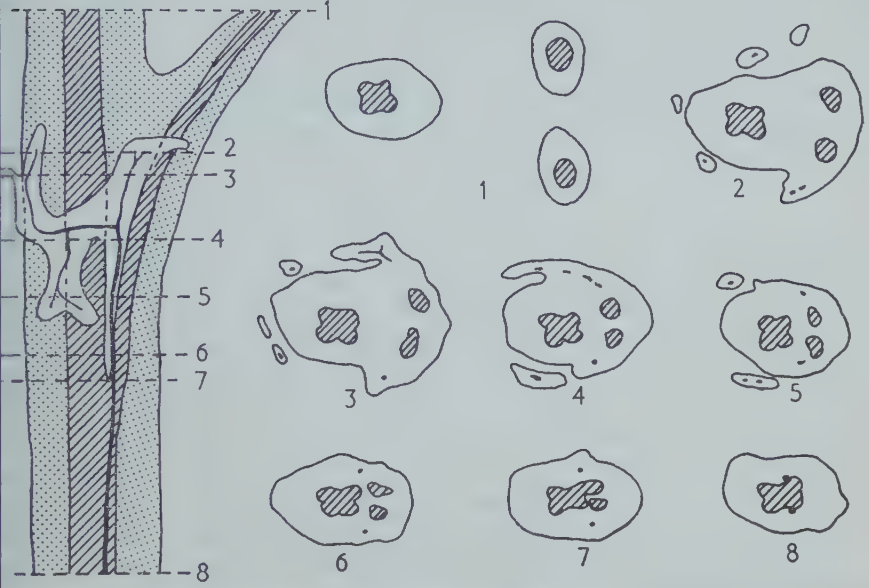
THE STRUCTURE OF THE APHLEBIAE

The three branching regions which were studied in detail showed considerable variation in the form of the aphlebiae. Some distortion has probably occurred before or during petrifaction, but all these aphlebiae are clearly more complex than simple forked processes.

In Text-fig. 1 some serial sections on which Model A (Pl. XXVIII, Figs. 2 and 3) is based are illustrated, and a reconstruction to show the vascular supply on one side of the node is given. From the size of the branches, judging by the measurements given by Surange (1952), it seems likely that these sections show the departure of 2nd-order branches from a 1st-order branch. This is confirmed by the origin of the aphlebia traces directly from the protoxylem of the main axis. Each aphlebia is joined to the surface of the 1st-order branch by an oblique ridge to the side of and somewhat below the level of insertion of the two 2nd-order branches, which are fused to each other at the base. The ridge bears appendages which differ in detail in the two members of the pair of aphlebiae. The aphlebia on one side shows five distinct appendages, four of which are a clear result of forking and the aphlebia on the other side bears four processes.

The aphlebiae at the other two nodes investigated were joined to the axes by a single protuberance rather than by an elongated ridge. Like the ridge, however, the protuberance bore a number of appendages of a forking nature. In Model B (Pl. XXVIII, Fig. 4), for example, the aphlebia is seen to bear seven appendages, one of which is almost 4 mm. in length.

Each large appendage has its own vascular supply, consisting of a simple strand of small scalariform elements. The traces of the aphlebiae arise from e stele of the parent branch or from the daughter branch, depending on the der of the branches (Surange, 1952). Serial sections show that this vascular pply branches in a dichotomous fashion (Text-fig. 1, No. 3, and reconstruc-



TEXT-FIG. 1. Serial sections through a branching region and a diagrammatic reconstruc- on showing the vascular supply (×9·2).

The aphlebia on the lower side of the transverse sections is illustrated in the reconstruction. he origin and mode of departure of the aphlebia traces are shown, as well as the branched ture of the aphlebiae. The vascular tissues of the main axis and its branches are shaded; the hlebia traces and vascular tissues in the branches of the aphlebiae are shown in solid black. Section No. 1 is at the top of Model A and No. 6 at its base.

on), forming strands running into all except the very small appendages. urange (1952) has noted a bifurcation of the aphlebia trace 'immediately fter coming out of the petiole', and one of his figures (Pl. 2, Fig. 15) shows suggestion of a second fork of the aphlebia. Further, a figure by Bertrand 1909, Pl. VIII, Fig. 59), reproduced by Hirmer (1927, p. 493, Fig. 586), hows clear evidence of branching of one of the aphlebiae of *S. burntislandica* n the basis of about four separate vascular strands. Bertrand (1907, 1909) as described the aphlebiae of *S. oldhamia* and *S. burntislandica* as laciniate tructures in the form of filamentous tufts, and also notes (1909, p. 54) that sually one of the branches of the aphlebia is directed horizontally towards he main axis. This feature, as well as the much-branched character of the phlebiae, has now been confirmed for *S. burntislandica* (see Pl. XXVIII, ig. 3).

The Nature of the Aphlebiae

A number of authors, including Scott (1920), Bower (1935), and Surange 1952), have expressed the view that the aphlebiae of *Stauropteris* are emergences or enations, the morphological equivalent of the *Thursophyton* leaves of *Asteroxylon*. On the other hand, Zimmermann (1938) and Lam (1948) suggest that aphlebiae may be of telome character. The constant angular position of these structures in *Stauropteris* and their branching nature and dichotomous vascular supply now demonstrated may be taken to afford some evidence, although not conclusive (cf. Browne, 1935), for the latter view. It seems possible that these aphlebiae, borne on fronds showing well-marked dichotomy, could themselves be interpreted in terms of dichotomies, but here condensed and overtopped.

THE MEGASPORANGIUM

Megasporangia are abundant in many of the sections prepared in this investigation. Most of these megasporangia contained two megaspores, the normal number (Surange, 1952), but some sporangia were seen in which there were three or four spores, an observation giving support to the findings reported by Surange (1952, p. 85). In one section through a megasporangium, however, at least five or six spores or spore prints were present (Pl. XXVIII, Fig. 5), and the high number of spores in this section was confirmed by the next peel of the series (Pl. XXVIII, Fig. 6). Examination of the spores of these serial sections reveals the presence of one clear triradiate cuticular mark, indicating one fairly large tetrad; evidence for the presence of several more spores, probably representing a second smaller tetrad at the apex of the megasporangium, is given in Pl. XXVIII, Fig. 5. One other megasporangium, less well-preserved than that described above, appears to contain six or more spores, in this case of approximately the same size. The rather swollen appearance of the distal end of these megasporangia as compared with those containing only two megaspores may be noted. On the present evidence it is suggested that a reduction in number of megaspores from a possible number of eight rather than from a single tetrad may sometimes have occurred.

ACKNOWLEDGEMENT

We are indebted to Mr. H. Joyner for preparation of the photographs.

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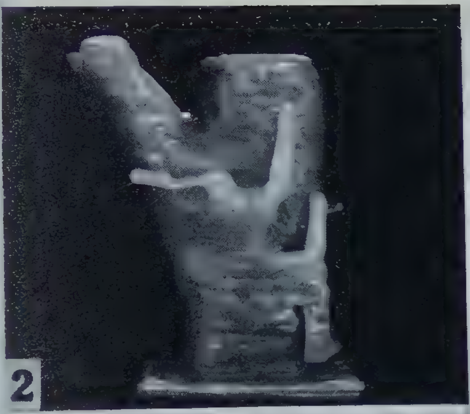
EXPLANATION OF PLATE XXVIII

Figs. 2 and 3. Two views of Model A showing the departure of the daughter branches and the insertion and form of the aphlebiae (× 9·2).

Fig. 4. Model B showing an aphlebia with a number of elongate appendages (× 9·2).

Figs. 1 and 6. Sections through a megasporangium containing an unusually high number of megaspores. In Fig. 5 about six spores or spore prints are indicated (× 87).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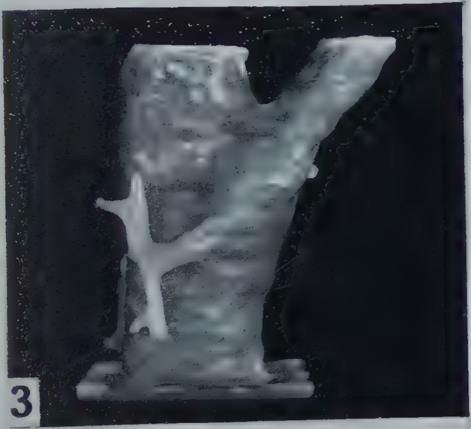
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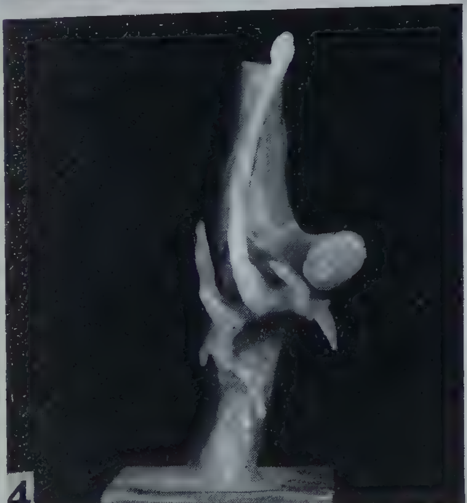
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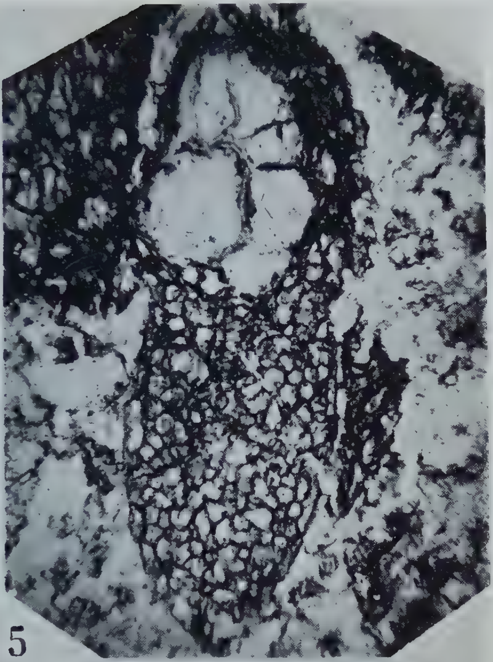
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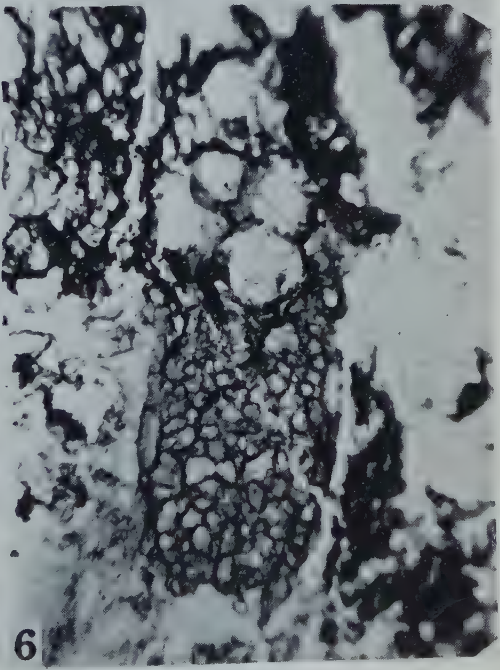
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W. S. LACEY, K. W. JOY, *and* A. J. WILLIS

Studies in the Regeneration of Horseradish

III. External Factors

BY

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With one Figure in the Text

ABSTRACT

Initiation of new primordia on a freshly-cut root is relatively insensitive to external conditions, and may take place under conditions that inhibit the later stages. The latter show a marked temperature optimum for both regeneration and respiration at 30° C. Moisture is essential, but complete immersion in water is inhibitory; oxygen is essential, and excess (6 per cent.) CO_2 without effect.

I. INTRODUCTION

THIS communication is essentially a pragmatic interlude between the last of the series (on correlation—Dore and Williams, 1956) and the next (on polarity); its primary purpose has been to ascertain the most suitable laboratory conditions for regeneration. A somewhat similar investigation has been carried out by Lindner (1940) in respect of temperature, pH, and the presence of a variety of substances in the external medium; we have re-examined the effect of temperature, since the material used (2-cm. long cuttings regenerating for the first time) differs greatly from that of Lindner (tissue-plugs from old roots), and have extended the investigation to include other factors, particularly humidity.

A secondary purpose has been to ascertain whether the different stages of regeneration respond differentially to external factors. Moreover, the rapid production of a large number of new apices can hardly fail to be reflected in the metabolism of the tissues concerned; and we have therefore made concomitant respiratory observations.

II. EXPERIMENTAL RESULTS

1. *The effect of temperature on regeneration*

Constant temperature. 2-cm. long cuttings (5 or 6 per Petri-dish) were grown on 2 per cent. plain agar in the dark in incubators maintained at 25°, 30°, and 35° C.; total primordia were counted daily, and a typical result is shown in Text-fig. 1*a*, in which regeneration is expressed as 'primordia per scar' (Dore, 1955). The 25° and 30° results confirm Lindner's (1940) statement that temperatures within the normal physiological range have little effect on initiation until the 6th–8th day; this is the time when differentiation

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of the primordia into buds or roots is at its most active, and the associated correlation effects on initiation of new primordia have been discussed by Dore and Williams (1956). The optimum under our conditions is clearly nearer 30° than 25°; the lower optimum reported by Lindner may be connected with the fact that his material was stored at low temperature (2°–4°) for a considerable time before use.

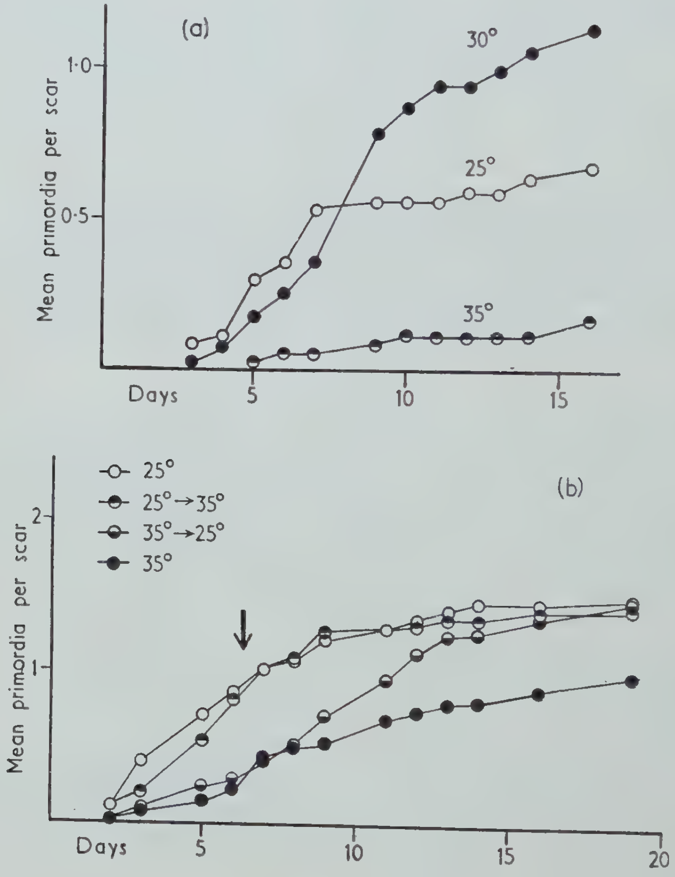

FIG. 1.

Change in temperature. Cuttings were subjected to four treatments: grown continuously at 25° and 35° C.; or grown for 6 days at one of these temperatures and then transferred to the other. The experiment was continued for 19 days, and the results are shown in Text-fig. 1*b*. The reduction in regeneration at 35° is not associated with any permanent damage; when the temperature is lowered to 25° initiation of new primordia takes place at the rate normal for that temperature. Measurements of bud- and root-length showed that extension growth is virtually completely inhibited at 35°, but again proceeds normally on transfer to 25°.

2. *The effect of temperature on respiration*

Roots not exceeding 5 mm. diameter were sliced and aerated in water for 24 hours before use; aeration was carried out both at room temperature and at the temperature of subsequent measurement. Respiration rates were measured by the Warburg direct method, readings being taken every 15 minutes. Since early experiments suggested an effect of dinitrophenol (DNP) on regeneration (which later experiments failed to confirm) a parallel series was carried out with added 5 per cent. DNP. The results are given in Table I, and we note the following points:

TABLE I

Respiratory Data (R.Q. and CO$_2$ output in μl/hour/gm. fresh weight) for Freshly-cut Horseradish Root Slices

Temperature (°C.)	Without added DNP				With 5% DNP			
	Aerated at room temperature		Aerated at experimental temperature		Aerated at room temperature		Aerated at experimental temperature	
	R.Q.	CO$_2$	R.Q.	CO$_2$	R.Q.	CO$_2$	R.Q.	CO$_2$
20	1·05	141	1·15	157	0·96	150	1·10	152
25	1·10	206	1·15	215	1·00	188	1·10	200
30	0·98	350	1·00	404	1·10	356	1·30	400
35	(1·5)	100	(1·8)	50	(2·0)	(140)	erratic	(300)
40	(3)	235	—	—	(2)	(120)	—	—

NOTES: (i) Figures in parentheses are approximate only.
(ii) Missing values at 40° represent death of tissue; no results were obtainable at 45° C.

(i) DNP at this concentration has little effect, as might be expected from its lack of effect on regeneration.

(ii) There is a marked optimum at 30° C., paralleling that for the post-initiation phase of regeneration.

(iii) The R.Q.s up to and including 30° C. are in good agreement and close to unity.

(iv) The CO$_2$-output is unusually high, and this tissue has consequently proved very useful for class experiments.

3. *Water requirements*

In the course of experiments to ascertain the most suitable medium for supporting the regenerating cuttings, a comparison between filter-paper and sand suggested that the medium was less important than the amount of moisture present. Experiments were therefore set up as follows: In Experiment 1, exactly equal quantities of water were added to two Petri-dishes, one containing sand and one filter-paper, the quantities of which were adjusted so that a thin film of water was just visible on each; in Experiment 2 the sand was much wetter than the filter-paper. Each experiment was duplicated. The experiments were carried out at different times, which produced an overall difference (probably a seasonal effect, *vide* Dore, 1953) irrelevant to this discussion. The results (for 4 and 11 days) are given in Table II.

TABLE II

Effect of Moisture: All Results Expressed as Mean Primordia per scar

		Occasion I			Occasion II		
		4 days		11 days	4 days		11 days
Experiment 1	Sand (wet)	0·140(5)	***	1·188(5)	0·525(6)	*	1·591(17)
	Filter-paper (moist)	0·858(5)		1·426(5)	0·982(6)		1·945(17)
Experiment 2	Sand (moist)	0·186(6)		0·572(6)	0·181(6)		0·600(6)
	Filter-paper (moist)	0·078(6)		0·658(6)	0·145(6)		0·555(6)

NOTES: (i) Numbers in brackets represent numbers of replicate cuttings.
(ii) Differences:
*** Difference significant at $P = 0{\cdot}001$ level.
* Difference significant at $P = 0{\cdot}05$ level.
In all other cases the values for each (sand/filter-paper) pair do not differ significantly.

It is clear that excess water markedly reduces primordium formation at first, though the numbers become equal after 11 days. This recovery is not due to loss of water, but nor is it necessary to ascribe it to some form of 'recovery' from inhibition. We already know that primordium-formation is checked when differentiation of existing primordia begins; this check in the less moist (and faster regenerating) dishes presumably allows the more slowly regenerating cuttings in the wetter dishes to attain parity. There was, incidentally, no disturbance of bud/root ratios, but extension growth of roots (not of buds) was noticeably reduced under the wetter conditions.

As might be expected from these results, experiment showed that complete immersion in water almost completely suppressed regeneration. (Cuttings that had been so immersed for 11 days nevertheless regenerated at normal speed when transferred to damp filter-paper.) In this respect our results differ from those of Beals (1923) and Balls and Hale (1934), both of whom reported regeneration under water. The discrepancy is likely to be due to their use of older tissue which, possessing as it does the ability to form callus, may well behave differently.

Further experiments using air of known different humidities showed clearly that no regeneration would take place below full saturation, and that below about 95 per cent. relative humidity the cuttings shrivel.

4. *Respiration and cutting age*

The respiration data of Table I are for freshly-cut roots before regeneration is detectable. Table III gives similar data for subsequent stages and we may note the increase in respiration when regeneration begins. (The slices do *not* include the regenerated buds and roots.) The falling-off in the later stages is probably connected in part with exhaustion of the starch reserves; rough estimations have shown that the starch falls from its initial value of 120 mgm. per gm. fresh weight to about 30–40 mgm. per gm. in 14 days, and the low R.Q. at 28 days probably reflects protein respiration. We have found that 21 days at 35° C. brings about complete destarching, though on subsequent transfer to 25° C. limited regeneration (a few buds only) still occurs.

TABLE III

Effect of Cutting Age on Respiration

Age in days	Physiological status	R.Q.	CO_2 output (μ^1/hour/gm. fresh weight)
0	Freshly cut	1·10	193
3	Initiation beginning	1·04	362
10	Early extension stage	1·15	297
14	Extension	0·96	75
28	Exhaustion of starch reserves?	0·79	erratic

5. *Oxygen and* CO_2

Differentiation and extension apparently do not occur in the absence of oxygen. After 14 days' growth in nitrogen a few primordia were initiated which remained undifferentiated; and although this cannot be taken as evidence of anaerobic initiation—we would not claim to have certainly removed every trace of oxygen from the system—it seems likely that the initiation stage is less oxygen-dependent than the others. The failure to regenerate under water may well also be due to lack of oxygen: *vide* also Wardlaw and Allsopp (1948) for a demonstration that oxygen is required for the growth of fern meristems. A moderate excess of CO_2 is not inhibitory; cuttings grown in air containing 6 per cent. CO_2 regenerated at the same rate as controls in CO_2-free air. There was, though, a barely significant increase in extension of roots.

III. DISCUSSION

Apart from their purely practical usefulness, the most striking feature of these results is the strength of the initiation stimulus. Not only (as was already known) is it relatively temperature-insensitive over the physiological range; there are suggestions that it will still occur under conditions—35° C. and lack of oxygen—completely inhibiting to the later stages. Primordium-initiation involves only rapid cell-division, and it is clear that, providing the apparatus for self-duplication is present, its metabolic requirements are negligible. The subsequent differentiation and extension necessarily involve rapid protein and cellulose synthesis, which in their turn will require the energy and carbon-skeletons of respiration. The 30° optimum for initiation after the 7th day is thus probably a secondary effect—the respiratory optimum controlling the extension of existing primordia, which in turn affects the initiation of further primordia.

ACKNOWLEDGEMENTS

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The Influence of Mannitol and Auxin on Growth of Cell Walls in *Avena* Coleoptiles[1]

BY

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With Plate XXIX and one Figure in the Text

ABSTRACT

Studies were made on the structure of cell walls in *Avena* coleoptile segments incubated in sucrose and buffer without IAA (indoleacetic acid), with 8 p.p.m. IAA, and with 8 p.p.m. IAA+0·2 or 0·3 M mannitol respectively. Examination in electron and polarizing microscopes revealed no difference in the structure of walls from segments grown in IAA and in IAA+mannitol. Measurements of the weight of wall material showed that in the presence of mannitol, wall deposition was inhibited. Measurements on the uptake of oxygen by segments showed that mannitol eliminated a respiratory stimulus produced by IAA. It is suggested that new wall material is only deposited on cell walls which are being thinned by elongation, and that the respiratory stimulus produced by IAA and eliminated by mannitol may be in part a manifestation of metabolism involved in the synthesis of new wall material. Wall extension does not appear to be dependent on deposition of new material.

INTRODUCTION

IN many studies on the physiology of cell growth, particularly with *Avena* coleoptiles, mannitol has been used to inhibit cell enlargement (Ordin *et al.*, 1955, 1956; Thimann and Schneider, 1938). It has generally been assumed that mannitol acts solely as an osmotic agent in preventing water uptake and has little direct effect on cell metabolism (Thimann, 1951, 1954; Thimann and Samuel, 1955). If this is so, it might be further assumed that while mannitol prevents increase in wall area it does not affect the synthesis and deposition of new wall material. This latter assumption should be capable of being tested experimentally.

Recent observations have suggested that the multi-net model of Roelofsen and Houwink (1953) most satisfactorily explains growth of walls of parenchyma cells in *Avena* coleoptiles (Wardrop, 1956; Setterfield and Bayley, 1957). According to this model, transversely oriented cellulose microfibrils are deposited on the interior of the wall, while elongation of the cell causes a progressive reorientation of the microfibrils already deposited. In the presence of mannitol, continued wall deposition without extension should therefore result in a general thickening of the wall with an increased number of transversely oriented microfibrils on the interior.

A test of this hypothesis could provide useful information on both the mechanisms of cell extension and the action of mannitol. To this end microscopic

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observations were made on cell walls of *Avena* coleoptile segments grown in the presence and absence of inhibiting concentrations of mannitol. The weights of cell walls from such segments were also measured. To learn whether mannitol interfered with metabolism under the conditions of these experiments, the uptake of oxygen by coleoptile segments was measured.

MATERIALS AND METHODS

The method of growing segments of *Avena* coleoptiles, based on that of Nitsch and Nitsch (1956), was as follows except for slight modifications in the weight and respiration experiments (q.v.). Segments 4 mm. long were cut 3 mm. below the tips of coleoptiles usually about 20 mm. long. After preliminary immersion in 1 p.p.m. $MnSO_4$ for 3 hours, the segments were transferred to the appropriate growth solutions at 23 ± 1° C. Up to ten segments were floated on 1 ml. of growth solution in a watch glass on a slow rotary shaker. The following growth solutions were used:

1. 2 per cent. sucrose in 0·01 M phosphate-citrate buffer, pH 5·0.
2. As (1) plus 8 p.p.m. IAA.
3. As (2) plus 0·2 M mannitol.

The growth period was always at least 20 hours, at the end of which the length of the segments was measured to the nearest 0·5 mm.

Microscopy. For electron microscopy, coleoptile segments grown in the above manner were cut into 1 to 2 mm. lengths and treated in one of the following ways:

1. Fixed in 70 per cent. ethyl alcohol, 4–12 hours.
2. Fixed in 2 per cent. osmic acid in 0·14 M veronal-acetate buffer, pH 7·4, 4 hours.
3. Treated with 10 per cent. pectinase for 6–8 hours; fixed as in (1) or (2).
4. Extracted for 2 hours in 1 N HCl at room temperature followed by 2 hours in 17·5 per cent. NaOH at room temperature. Fixed as in (1).

Following fixation, the tissue was dehydrated, embedded in methacrylate and sectioned on a Porter-Blum microtome. Embedding medium was removed from sections and they were shadowed with palladium-gold (Bayley *et al.*, 1957). It may be noted here that wall structure appeared the same after either osmic acid or alcohol fixation.

For polarizing microscopy, segments were extracted in hot 0·05 N HCl for 1 hour followed by 1 hour in 17·5 per cent. NaOH at room temperature. They were then squashed beneath coverslips in water on microscope slides. The optical path differences through individual parenchyma cells were measured at 5,460 Å using a rotating $\lambda/12$ mica plate in the substage of a polarizing microscope.

Weight measurements. For these, the initial length of the segments was increased to 8 mm. and the concentration of mannitol to 0·3 M. Groups of 30 segments, cut from coleoptiles not longer than about 30 mm., were incubated with slow shaking for about 24 hours on 8 ml. of growth solution in 50-mm.-

diameter Petri-dishes. After the appropriate growth treatment, the lengths of the segments were measured, the primary leaves removed and the segments rinsed in distilled water and ground with mortar and pestel. The resulting fragments were washed three times in distilled water with centrifuging, dried *in vacuo* at 60° C. overnight, and weighed. The residues were then extracted in hot 0·05 N HCl for 1 hour followed by hot 0·5 per cent. ammonium oxalate for 1 hour. After washing, the residues were dried as before and reweighed. Finally they were extracted with 17·5 per cent. NaOH for 4 hours at room temperature, washed, dried, and reweighed.

Respiration measurements. Uptake of oxygen by coleoptile segments initially 8 mm. long, grown without and with IAA and with IAA+0·3 M mannitol respectively, was measured in a Warburg respirometer. Eight segments less primary leaves were assigned to each flask and duplicate flasks were used for each growth solution. Incubation was at 24·8° C. in the dark. Oxygen uptake was measured by Warburg's direct method using 3 ml. of growth solution and 0·2 ml. NaOH in the centre well. At the end of the experiment, the lengths of the segments were measured.

RESULTS

Growth of segments. Table I summarizes the results of two experiments on the growth, under a variety of conditions, of coleoptile segments initially 4 mm. long. It will be noted that 0·2 M mannitol almost completely inhibited elongation. Upon removal from mannitol, segments showed a partial recovery in growth although their final length did not reach that of segments

TABLE I

Growth of Segments of Avena *Coleoptiles Initially 4 mm. long*

	Time in growth solution*—hrs.			
No. of segments	2% sucrose	2% sucrose +8 p.p.m. IAA +0·2M mannitol	2% sucrose +8 p.p.m. IAA	Mean length-mm.
5	20	—	—	5·5
6	—	—	20	9·6
9	—	—	24	10·2
3	—	—	46	11·2
6	—	20	—	5·0
2	—	24	—	5·0
21	—	40	—	6·3
9	—	46	—	6·4
8	—	24†	24	7·5
6	—	24†	45	7·9
6	—	48†	24	6·9

* All growth solutions were buffered at pH 5·0 with 0·01 M phosphate-citrate buffer.
† Grown first in IAA+mannitol then transferred to IAA alone.

grown entirely without mannitol. The segments from these experiments were used for microscopy.

Microscopy. In the electron microscope, comparison of wall structure in coleoptiles receiving different treatments was hampered by marked variations in wall thickness within the same coleoptile. Cells from different regions of the coleoptile possess walls of different thickness while the walls of individual parenchyma cells vary considerably in thickness from the junction of neighbouring cells to the mid-point between junctions. In comparing cells from differently treated coleoptiles, therefore, it was necessary to choose the same parts of cell walls from the same regions of the coleoptiles.

Figs. 1 and 2 (Pl. XXIX) are low-magnification electron micrographs of transverse sections of inner epidermal cells from coleoptile segments grown with IAA and IAA+mannitol respectively. They have been extracted with HCl and NaOH and the non-cellulosic material is partially removed. At this magnification the walls of the two cells appear almost identical.

Figs. 3 and 4 (Pl. XXIX) are views at a higher magnification of typical transverse sections of parenchyma walls near the mid-point between cell junctions, from coleoptile segments grown with IAA and IAA+mannitol respectively. These walls were fixed without prior extraction and the cellulose microfibrils are completely obscured by encrusting non-cellulosic wall material. Longitudinal sections from segments treated as those in Figs. 3 and 4 are shown in Figs. 5 and 6 (Pl. XXIX). In both cases no obvious difference in thickness or structure between walls of cells grown in IAA and IAA+mannitol is present.

The microfibril arrangement in parenchyma walls is revealed in Figs. 7 and 8 (Pl. XXIX), which represent transverse sections of coleoptile segments grown in IAA and IAA+mannitol respectively and extracted with pectinase. The microfibril arrangement is similar in both and similar to that described previously (Setterfield and Bayley, 1957) for intact coleoptiles. On the inside, microfibrils run transversely while on the outside they are disoriented.

Under the polarizing microscope, parenchyma cells from coleoptile segments grown for 20 hours in IAA and IAA+mannitol respectively were negatively birefringent with respect to the cell axes. The mean optical path difference for 25 cells in IAA was 58±7 Å and for a similar number in IAA+mannitol was 50±6 Å, indicating no significant difference between the two groups.

Weight measurements. Two separate experiments were carried out and the results are summarized in Table II. Although the weights on a unit basis in the second experiment were somewhat lower than in the first, the differences resulting from different treatments were consistent in both experiments. The most important result is that the weights per segment in IAA+mannitol were markedly lower at all stages of extraction than those without and with IAA. Furthermore, for the material extracted with cold water, that is, whole cell wall (Thimann and Bonner, 1933), the weights per segment were roughly proportional to the final lengths of the segments.

Two further small but statistically significant differences emerge. First, the

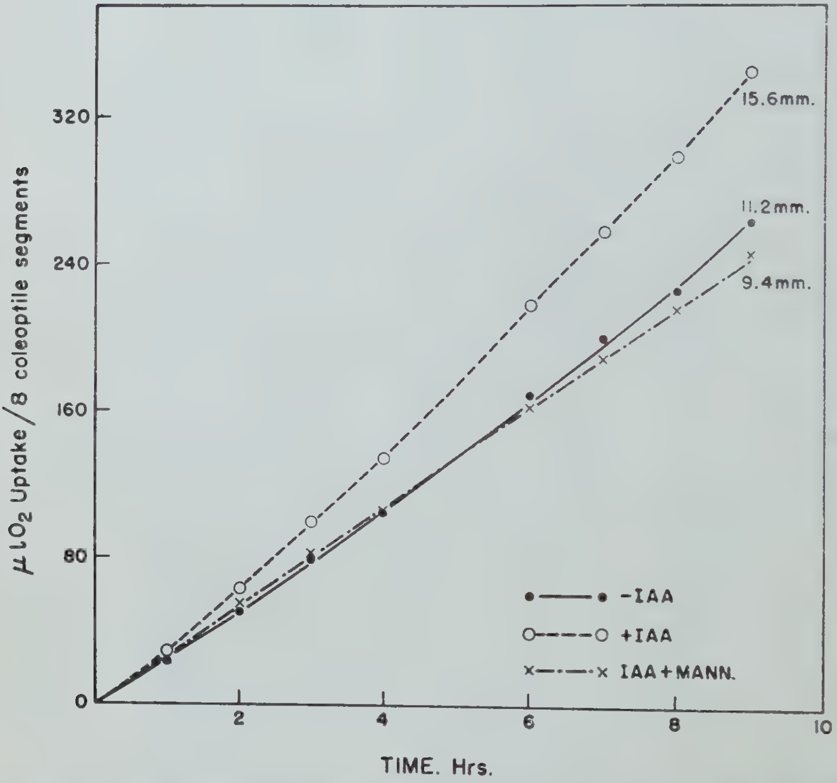
TABLE II

Dry Weights of Segments of Avena *Coleoptiles, initially 8 mm. long, grown under different conditions for 24 hrs.*

	Growth solutions*	No. of segments	Mean† length of segments mm.	Extraction procedures											
				Cold Water				0·05N HCl + 0·5% NH_4-oxalate				17·5% NaOH			
				Total wt.- mg.	Wt./ segment- mg.	Wt./ 100 mm.- mg.	%‡	Total wt.- mg.	Wt./ segment- mg.	Wt./ 100 mm.- mg.	%‡	Total wt.- mg.	Wt./ segment- mg.	Wt./ 100 mm.- mg.	%‡
Experiment 1	2% sucrose .	250	11·7	74·4	0·305	2·54	100	36·4	0·146	1·24	49	18·4	0·074	0·63	25
	2% sucrose . +8 p.p.m. IAA.	242	16·0	85·3	0·353	2·21	100	38·4	0·159	0·99	45	16·5	0·068	0·43	19
	2% sucrose +8 p.p.m. IAA. +0·3 M mannitol	244	9·8	58·7	0·235	2·45	100	30·3	0·124	1·27	52	13·2	0·054	0·55	23
Experiment 2	2% sucrose	372	12·1	85·4	0·230	1·90	100	45·0	0·121	1·00	53	22·3	0·060	0·50	26
	2% sucrose +8 p.p.m. IAA.	376	16·2	105·6	0·281	1·74	100	54·5	0·145	0·90	52	24·1	0·064	0·40	23
	2% sucrose . + 8 p.p.m. IAA. + 0·3 M mannitol	377	9·7	76·7	0·203	2·10	100	40·1	0·106	1·10	52	16·3	0·043	0·45	21

* All growth solutions were buffered at pH 5·0 with 0·01 M phosphate-citrate buffer.
† At end of 24-hour growth period.
‡ Weights following cold-water extraction taken as 100 per cent.

weights per 100 mm. for segments grown in IAA were slightly lower at all stages of extraction than those grown without IAA and with IAA+mannitol. A decrease of this type of about 5 per cent. is expected since the separation of end-walls of cells will be proportional to elongation (Preston and Clark, 1944). However, the mean differences for both experiments were actually 12 per cent.



TEXT-FIG. 1. Graph showing microlitres uptake of oxygen per 8 coleoptile segments (means of two flasks with 8 segments each) plotted against time. —IAA represents 2 per cent. sucrose; +IAA, 2 per cent. sucrose+8 p.p.m. indoleacetic acid; IAA+MANN, 2 per cent. sucrose +8 p.p.m. IAA+0·3 M mannitol; all in 0·01 M phosphate-citrate buffer, pH 5·0. The initial length of all segments was 8 mm.; figures in mm. give mean lengths of segments at the end of the experiment.

for material extracted with cold water and 25 per cent. for alkali-extracted material, indicating that thinning of the walls occurred during elongation. Second, the material remaining after alkali extraction (i.e. largely α-cellulose), expressed as a percentage of the cold-water-extracted material, was significantly higher for segments grown without IAA than with IAA and IAA+ mannitol.

Respiration measurements. Oxygen uptake of coleoptile segments grown without IAA, with IAA and with IAA+0·3 M mannitol is shown in Text-fig. 1. Over a 9-hour period, segments grown with IAA showed a noticeably higher rate of oxygen uptake than those grown without IAA. This is in agreement with the reports by other authors (Commoner and Thimann, 1941; Bonner, 1949) of respiratory stimulation by IAA. The presence of 0·3 M mannitol, on the other hand, completely eliminated this stimulation. Bonner *et al.* (1953) and Ordin *et al.* (1956) have reported a similar effect of mannitol in Jerusalem artichoke and coleoptile tissues respectively.

DISCUSSION

The results show clearly that in the presence of mannitol, no detectable deposition of wall material takes place. In the electron microscope unextracted walls from segments treated in mannitol showed no increased thickness or folding. After pectinase treatment there was no visible increase in the number of transverse microfibrils and in the polarizing microscope the optical path difference was not detectably changed. Finally, the weight of wall material from segments treated in mannitol was much less than in the controls and was of the order expected if no wall deposition had occurred.

It is apparent that besides inhibiting cell elongation, mannitol also prevents synthesis of wall material. Two possible explanations for this suggest themselves. First, besides osmotically inhibiting water uptake by the cell, mannitol may interfere directly with the metabolism involved in deposition of new wall material. Alternatively and more probably, mannitol may merely inhibit uptake of water by the cell and this in turn prevents cell elongation. Wall deposition would be inhibited as well if it is assumed that extension of the cell wall is necessary for deposition to take place; that is, if fresh wall material is deposited only on walls which are being thinned by stretching. This dependence of wall synthesis on thinning of the wall during cell elongation would be an important aspect of the growth of cell walls which at present is unexplained.

The possible dependence of wall synthesis upon extension would partially explain the respiratory stimulation produced by IAA and its elimination by mannitol. By promoting stretching and thinning of the wall, possibly by increasing wall plasticity (Heyn, 1940; Cleland and Bonner, 1956), auxin would indirectly cause increased wall synthesis and deposition which would be accompanied by increased respiration. Mannitol by inhibiting water uptake could indirectly eliminate this respiratory increase by merely preventing wall thinning.

At any rate, the fact that deposition of cell-wall material does not take place in the presence of inhibitory concentrations of mannitol suggests that studies on cell growth employing this compound must be interpreted with care since metabolism is obviously disturbed. Furthermore, inhibition of cell elongation by mannitol cannot provide a test of the multi-net model of wall growth as suggested by Wardrop (1956).

Instead of suggesting that wall extension is necessary for deposition, the evidence presented above might be taken to support the concept that cell-wall extension is dependent on and brought about by wall deposition, perhaps through active intussusception of new material. On the basis of the multi-net model, where new wall is added by apposition, this would appear unlikely. Further, the finding that the weight of wall per unit length decreases slightly in rapidly elongating segments (grown in IAA) indicates that extension can occur without exactly compensating deposition. Thus, although extension may be necessary for deposition, deposition may lag behind extension. This conclusion is supported by the finding of Preston and Clark (1944) that the weight of wall per unit length decreases during growth of intact coleoptiles. In addition, Bennet-Clark (1956) reports that in coleoptile segments grown without external supplies of sugar, cell elongation can take place without appreciable wall synthesis.

Bonner (1934) also concluded that wall deposition does not bring about extension, but it should be noted that results of the weight experiments presented here are somewhat at variance with his findings. Rather than a small thinning of wall as found here and by Preston and Clark (1944), this author found that in segments grown at 25° C. with IAA and fructose a small increase in wall weight per unit length occurred. Both growth and weight increases were very small in these experiments and it is possible that formation of secondary wall introduced a complication.

One other interesting fact emerges from the weight experiments, that is the small but significant increase in percentage of cellulose in the walls of segments grown without IAA. This suggests that more noncellulosic material may be deposited in the wall in the presence of IAA and lends support to the finding of Ordin *et al.* (1955) that IAA influences the metabolism of the noncellulosic wall fraction.

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EXPLANATION OF PLATE XXIX

Electron micrographs of cell walls in *Avena* coleoptile segments. Unlabelled arrows point toward the inside of cells. Scale lines represent 1μ.

Fig. 1. Segment grown 24 hours in IAA. Transverse section of an inner epidermal cell extracted with HCl and NaOH. Thickened wall on right borders on inner cavity of coleoptile. (×2,700.)

Fig. 2. As in Fig. 1 except that the segment was grown in IAA+mannitol. (×2,700.)

Fig. 3. Segment grown 24 hours in IAA. Transverse section of a parenchyma cell wall fixed in 70 per cent. EtOH without extraction. (×14,000.)

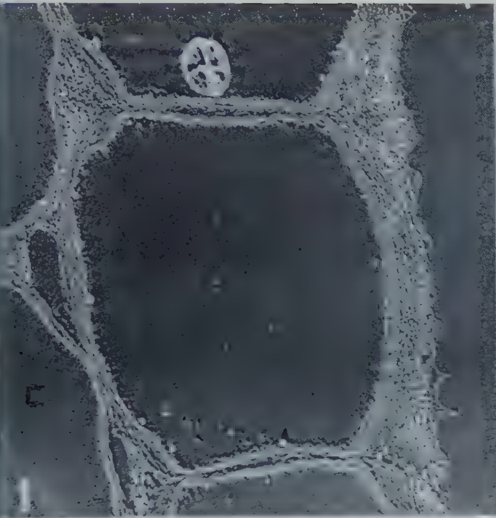
Fig. 4. As in Fig. 3, except that the segment was grown in IAA+mannitol. (×14,000.)

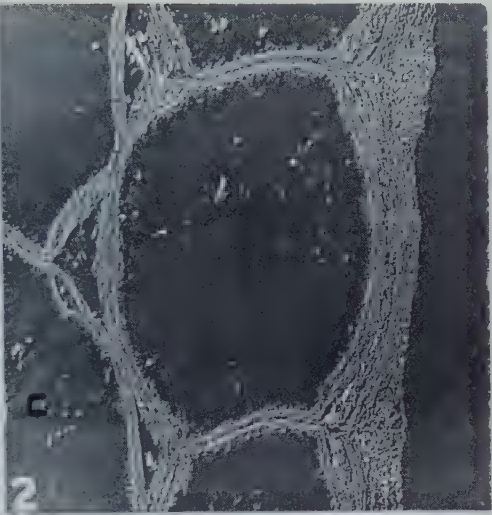
Fig. 5. As in Fig. 3. Longitudinal section. (×14,000.)

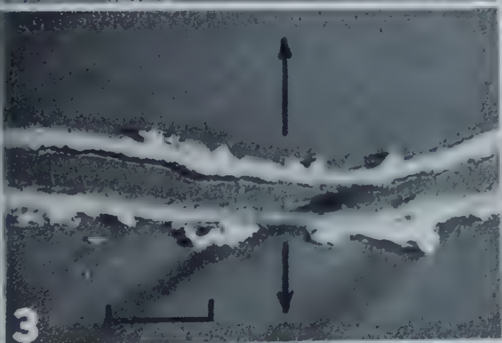
Fig. 6. As in Fig. 4. Longitudinal section. (×14,000.)

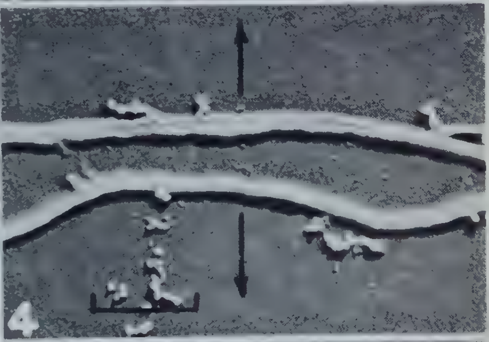
Fig. 7. Segment grown 24 hours in IAA. Transverse section of a parenchyma cell wall treated with pectinase before fixation. (×21,000.)

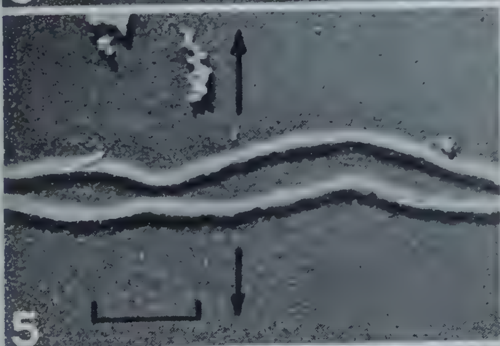
Fig. 8. As in Fig. 7, except that the segment was grown in IAA+mannitol. (×17,500.)

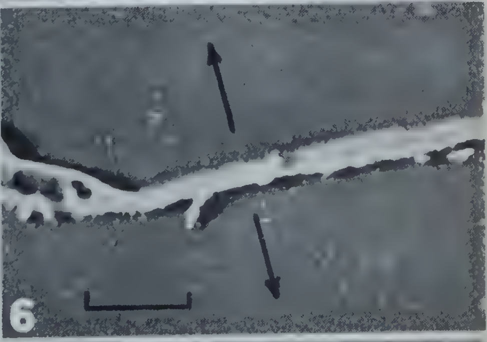


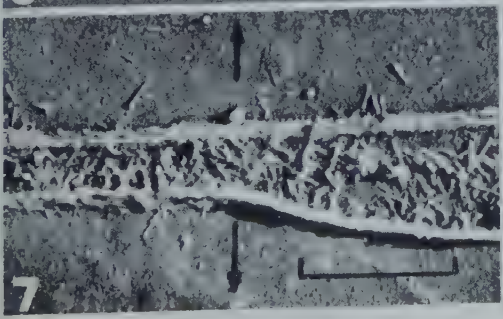


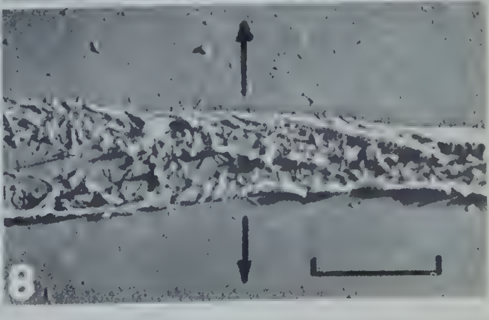












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